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(54) Title: METHOD FOR DETECTING GROWTH AND STRESS IN PLANTS AND FOR MONITORING TEXTILE FIBER QUALITY

(57) Abstract

A method of detecting environmental stress in plants, particular water stress in cotton plants is based on a hot dilute acid extraction of plant tissues such as cotton fibers. The extracts are analyzed by high pH anion exchange chromatography to separate and characterize the carbohydrates. This method extracts a characteristic series of carbohydrate multimers containing galactose, mannose and glucose. The pattern of multimers is indicative of growth stress during the formation of the plant tissue. In addition, similar multimers can be extracted from textiles and are indicative of textile wear and can be used to determine which manufacturing treatment will improve fabric life. In addition the multimers are shown to contain a protein component. Chemical agents that cross–link the protein component alter the extractability of the multimers. Thus, cross–linking of this type can be used to alter favorably the resistance of fabric to washing induced wear. Finally, a sequential enzymatic extraction method for producing high purity cellulose is disclosed.

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METHOD FOR DETECTING GROWTH AND STRESS IN PLANTS AND FOR MONITORING TEXTILE FIBER QUALITY

BACKGROUND OF THE INVENTION

The present application is a Continuation In Part of: Application 09/0003,679, filed January 7, 1998, which is a Continuation In Part of Application Serial No. 08/516,953, filed on August 18, 1995, now issued as U.S. Patent No. 5,710,047, January 20, 1998; of U.S. Provisional 60/096,162, filed August 11, 1998; and of U.S. Provisional Patent 60/106001, filed October 28, 1998, all of which are hereby incorporated by reference into this application.

1. Field of the Invention

This invention concerns a method of detecting environmental stress in green land plants, particularly in agricultural crops, so that production can be optimized by alleviating the stress before permanent damage to the plants occurs. In particular this application describes biochemical methods of assessing quality of cotton fibers.

2. Description of Related Art

In the parent of this application the present inventor described his surprising discovery that it was possible to extract a carbohydrate-containing fraction from properly prepared plant material by a simple cold water process. Essentially, plant tissue is prepared by rapid freezing (preferably by use of liquid nitrogen or solid carbon dioxide) and is then lyophilized and stored at temperatures below freezing. As disclosed in the above-referenced parent application carbohydrate-containing cell wall fractions can be easily extracted from the lyophilized tissue by cold aqueous extraction; then, greatly improved techniques of High Pressure Liquid Chromatography (HPLC)

allow resolution of the aqueous extract into constituent mono and polysaccharides which can be further hydrolyzed to identify the constituent monosaccharides.

The use of high pH anion exchange chromatography (HPAEC) makes possible the unambiguous identification of cell wall constituents. In HPAEC a salt gradient (such as a sodium acetate gradient) is applied to a column of special ion exchange resins held at a high pH to sequentially elute various mono and polysaccharides. Essentially, the hydroxyl groups of the sugars act as extremely weak acids which become deprotonated at the high pH, binding to the ion exchange matrix until eluted by the gradient.

While there are a number of vendors of HPAEC materials, the current invention has employed products and systems produced by the Dionex Corporation of Sunnyvale, California. These products and systems are explained in full in the Dionex Technical Notes, particularly in Technical Notes 20 and 21, which are hereby incorporated into this application. The carbohydrate fractions isolated from plant cell walls were analyzed using Dionex CarboPac PA1 and PA-100 columns. Both of these columns contain polystyrene/divinylbenzene cross-linked latex microbeads (350 nm diameter) with quaternary amine functional groups. The columns were operated under the manufacturer's recommended pressure conditions (4000 psi maximum) in sodium hydroxide eluted with a sodium acetate elution gradient. When necessary, sugar alcohols were analyzed using a CarboPac MA1 column which contains porous beads (8.5 μm diameter) of vinylbenzene chloride/divinylbenzene with alkyl quaternary ammonium functional groups.

The polysaccharides analyzed in the present invention are appropriately referred to as "glycoconjugates" because they comprise a monosaccharide *conjugated* to at least one additional monosaccharide (i.e., to form an oligo or polysaccharide) and optionally to a protein or a lipid. As will be disclosed below at least some of the glycoconjugates comprise polysaccharides conjugated to a protein moiety. To summarize glycoconjugates may be polysaccharides, polysaccharides containing a

protein moiety, polysaccharides containing a lipid moiety and/or any combination of these. In the present application only polysaccharides and polysaccharides containing a protein moiety have been unambiguously identified. In any case HPAEC characterizes the polysaccharide component of the glycoconjugate.

In the parent application two groupings of polysaccharides were especially pointed out and described by their position in HPAEC separations; these groups were identified as GC-1 and GC-2. Herein the composition of these groupings is further elucidated and other important polysaccharides (glycoconjugates) are discussed.

BRIEF DESCRIPTION OF THE DRAWINGS

The objects and features of the present invention, which are believed to be novel, are set forth with particularity in the appended claims. The present invention, both as to its organization and manner of operation, together with further objects and advantages, may best be understood by reference to the following description, taken in connection with the accompanying drawings.

Figure 1 shows an HPAEC of a cold aqueous extract of cotton fibers according to the present invention indicating the position and identity of a number of carbohydrates;

Figure 2 shows a typical HPAEC cold aqueous extract to show the position of the GC-1 and GC-2 carbohydrates;

Figure 3 shows an alkaline degradation experiment on multimers extracted from plant tissues according to the present invention;

Figure 4 shows the effect of incubating the cold aqueous extract with normally grown fibers wherein insoluble enzymes cause changes in the carbohydrate profile (arrows); the control uses fibers boiled to destroy the enzymes;

Figure 5 shows the effect of incubating the cold aqueous extract with drought grown (no irrigation) fibers wherein insoluble enzymes cause changes in the carbohydrate profile (arrows); the control uses fibers boiled to destroy the enzymes;

Figure 6 shows cold aqueous extracts from fibers taken at different times of day (early [A], mid [B], and late [C]) to illustrate that the carbohydrate pattern varies predictably with time of day;

Figure 7 shows the effect on the carbohydrate profile of incubating normally grown cotton fibers (harvested according to the method of the invention) with a number of different added substrates: A) control-boiled to destroy enzymes; B) inositol added; C) glycerol added; D) sucrose added; and E) inositol, glycerol, sucrose and arabinose added;

Figure 8 shows the effect on the carbohydrate profile of incubating drought stressed (non-irrigated) cotton fibers (harvested according to the method of the invention) with a number of different added substrates: A) control-boiled to destroy enzymes; B) inositol added; C) glycerol added; D) sucrose added; and E) inositol, glycerol, sucrose and arabinose added;

Figure 9 shows carbohydrate multimers extracted by HCl according to the present invention from cotton fibers ranging from 15 to 39 days post anthesis; these multimers are from cotton bolls of a normally grown plant and exhibit an extremely regular periodic pattern;

Figure 10 shows carbohydrate multimers extracted by HCl according to the present invention from cotton fibers ranging from 12 to 36 days post anthesis; these multimers are from cotton bolls of a stunted plant growing in a portion of the field receiving suboptimal irrigation and exhibit an irregular pattern particularly between 15 and 20 minutes of retention;

Figure 11 compares multimers extracted from a normal cotton fiber with multimers extracted from portions of sugar beet root to demonstrate that some of these carbohydrates are found in cell walls of widely divergent plants; here the sugar beet tissue has been infected by a fungus (*Rhizoctonia*); the stress of infection alters the sugar beet multimers;

Figure 12 shows multimers extracted from normal (p) and abnormal "white speck" (w) fibers;

Figure 13 shows an enlarged view of the multimer profile from Figure 12 showing that the white speck (w) fibers have increased arabinose (ara) over the normal (p) fibers;

Figure 14 shows the multimers extracted from normal fibers after incubation with a number of different substrate combinations (identified in Table 1);

Figure 15 shows the multimers extracted from an undyed (A) and dyed (B) cotton towel; in each case the top trace is an extract of the new towel and the bottom trace is an extract after one laundering;

Figure 16 shows multimers extracted from an old, much laundered pillowcase (top) and towel (bottom);

Figure 17 shows a flow diagram for a proteolytic enzyme experiment with early, midday and late cotton fibers;

Figure 18 shows the multimers extracted using the scheme of Figure 17 for early (A), midday (B) and late C) fibers; besides the carbohydrate multimers, protein is also shown (A_{280}); and

Figure 19 shows the three multimer extracts of Figure 18 treated with protease: trypsin (T), chymotrypsin (CT) or no protease control (C).

Figure 20 shows the effect of boiling the cotton fibers before extracted the glue matrix.

Figure 21a shows the boiled versus non-boiled extractions as in Fig. 20 performed on fibers harvested at noon.

Figure 21b shows the boiled versus non-boiled extractions as in Fig. 20 performed on fibers harvested in the morning.

Figure 22 shows the results of acid hydrolysis of the glue matrix extracted in Fig. 20.

Figure 23 shows the composition of extracted glue matrix either passed (filtrate) or retained (retentate) by various molecular weight cutoff filters; from top to bottom: 30 kilodalton filter retentate; 10 kilodalton filter retentate; 4 kilodalton filter retentate; and 4 kilodalton filter filtrate.

Figure 24 shows the composition of extracts from 8 DPA fibers collected at three time (morning, noon or evening) and extracted at one of three temperatures: 37°C, 25°C or 4°C.

Figure 25 compares the carbohydrate composition of fibers with "white flakes" associated with the same fibers.

Figure 26 shows the effects of incubating various substrates (CB=cellobiose; raf=raffinose) with fibers during the extraction process and the effect of added tunicamycin (T) on the process.

Figure 27 shows the effect of cellulase or β-glucosidae on isolated multimers; control is the isolated multimers without enzyme treatment.

Figure 28 shows the carbohydrates extracted from the first incubation of fibers treated with protease first (chymotrypsin) or cellulase first; PMSF=phenylmethylsulfonyl fluoride, a serine protease inhibitor.

Figure 29 shows the carbohydrates released from the second incubation of the fibers; the cellulase fibers had a first incubation with chymotrypsin and the chymotrypsin fibers had a first incubation with cellulase.

Figure 30 shows the multimers extracted from the fibers following the two extraction of Figs. 28 and 29; CT=chymotrypsin, PMSF=phenylsulfonyl fluoride.

Figure 31 shows carbohydrates released by cellulase alone (1) or following crosslinking with either 125 mM (2) or 250 mM (3) carbodiimide.

Figure 32 shows carbohydrates released by chymotrypsin alone (1) or following crosslinking with either 125 mM (2) or 250 mM carbodiimide (3).

Figure 33 shows carbohydrates released by a cellulase treatment (following a first chymotrypsin treatment) alone (1) or following crosslinking with either 125 mM (2) or 250 mM (3) carbodiimide.

Figure 34 shows carbohydrates released by a chymotrypsin treatment (following a first cellulase treatment) alone (1) or following crosslinking with either 125 mM (2) or 250 mM (3) carbodiimide.

Figure 35 shows the absorbance at 280 nm of carbohydrates released by chymotrypsin indicating the presence of a protein or glycoprotein.

Figure 36 shows the hydrolysis products of the white particle (presumably cellulose) left following the enzymatic digestions of Figs. 31-34.

DETAILED DESCRIPTION

OF THE PREFERRED EMBODIMENTS

The following description is provided to enable any person skilled in the art to make and use the invention and sets forth the best modes contemplated by the inventor

of carrying out his invention. Various modifications, however, will remain readily apparent to those skilled in the art, since the general principles of the present invention have been defined herein specifically to provide methods for determining plant growth stress and the quality of plant materials, especially cotton fibers, through the analysis of certain polysaccharide fractions.

As already discussed, glycoconjugates are carbohydrates covalently linked to other carbohydrates, proteins or lipids. The glycoconjugates monitored in the present study appear to function as cell wall precursors or intermediates in the biosynthetic processes that produce the cell wall. Cotton fibers are unique as plant cells in that their primary function is the synthesis of cell wall material. A progression of appearance and disappearance of specific glycoconjugates has been observed in developing cotton fibers under "normal" conditions. Developing cotton fibers obtained from plants subjected to various forms of stress, which negatively impacted fiber development, demonstrate an abnormal or altered pattern of appearance and disappearance of the glycoconjugates monitored. Glycoconjugate analysis is a sensitive method of monitoring cell wall synthesis which is directly coupled with cell growth. This analysis is applicable to roots, stems, leaves and fruits. In the present case, the analysis has been applied to a fruit. The presence of these glycoconjugates has been demonstrated in a range of different plants which leads to the conclusion that they will be found in virtually all plant cells. In addition to the monitoring of fiber growth and development, glycoconjugate analysis will demonstrate the presence of trehalulose or melizitose, oligosaccharides present in whitefly honeydew, if they are present. Thus, the method is also useful for monitoring insect pests.

Sucrosyl Oligosaccharides (GC1 Glycoconjugates)

The GC-1 series of glycoconjugates described in detail in the parent application is shown here to be composed of molecules in the raffinose series of oligosaccharides also known as the sucrosyl oligosaccharides. Raffinose is a nonreducing trisaccharide

consisting of D-galactose, D-glucose and D-fructose with the galactose and glucose linked by an α-1,6 glycosidic bond, and the fructose linked to the glucose by an α, β-1,2-glycosidic bond. That is, raffinose comprises a galactose unit linked to sucrose (glucose + fructose). It is believed that raffinose is synthesized by transferring a galactose unit from galactinol (dulcitol) to sucrose. Galactinol is produced by transferring a galactose unit from UDP-galactose to *myo*-inositol. Successive members of the raffinose series (stachyose, verbascose, and higher homologs) are produced by stepwise addition of galactose units. Thus, stachyose has two galactose units (galactobiose) added to sucrose, and verbascose has three, etc. At each step a galactinol molecule yields one galactose unit and one free *myo*-inositol molecule. Related sugars include melibiose (galactose + glucose) and manninotriose (galactose + galactose + glucose).

Until now the majority of research on the sucrosyl oligosaccharides has focused on their synthesis and apparent role as storage products in seeds. However, it is likely that these oligosaccharides serve as glycosyl donors for polysaccharide synthesis in cell walls (e.g., cotton fibers). There is a large body of literature describing cell wall invertases in a wide variety of plants. However, no obvious function for cell wall invertases has been proposed other than for cells in suspension culture. One report of the invertases in developing cotton fibers (Bucala, 1987) compared the activity of the cell wall invertases on sucrose, raffinose and stachyose. As might be expected from kinetic considerations, the activity decreased with increasing molecular weight. It seems likely that the cell wall (insoluble) invertases are cleaving fructose from the raffinose series compounds and transferring these sugars to other carbohydrates that comprise part of the complex cell wall structure. In one experiment it was possible to verify that the insoluble invertases convert verbascose (galactose, galactose, galactose, sucrose) to verbascotetrose (galactose, galactose, galactose, glucose). Presumably the cleaved fructose is added to some other carbohydrate.

Earlier GC-1 compounds (**14**) in Fig. 2 were defined as a group of carbohydrates that run at around 15 minutes in the standard separations described in the standard separations disclosed in the parent application. Fig. 1 shows a portion of a chromatogram similar to that of Fig. 2 except that the various carbohydrate peaks have been identified through hydrolysis experiments and through running known standards. Significantly the GC-1 compounds are identified as raffinose, stachyose and verbascose. Preliminary results indicate that samples with additional GC-1 peaks (e.g., Fig. 2) have additional higher homologs of the raffinose series. The significant point is that "raffinose series carbohydrates" is a more accurate term that can be substituted for GC-1 carbohydrates in the methods of the parent application. From quantitative interrelationships shown in the parent application GC-1 carbohydrates are believed to be precursors to the GC-2 carbohydrates. However, whereas raffinose series carbohydrates are nonreducing, current experiments have shown that the GC-2 carbohydrates are reducing. Thus, GC-1 compounds are probably not directly converted into GC-2 compounds. Rather carbohydrates are probably transferred (perhaps by the agency of a cell wall invertase) to other carbohydrate molecules to form GC-2 compounds.

An extremely exciting and unexpected discovery of the present study is that the synthetic enzymes necessary to make the GC-1 to GC-2 interconversions (and presumably other steps of cell wall synthesis) are preserved by lyophilization. If the cold aqueous extract is allowed to react with the fibers (which contain the nonsoluble enzymes), the carbohydrate profile of the extract changes with time as simple sugars are used up and more complex carbohydrates appear in their place. Fig 4 shows the results of incubating a cold aqueous extract of normally grown cotton with the fibers from which the extract was made. Fig. 4a shows the control (i.e., the aqueous extract) while Fig. 4b shows the results of the incubation in which the aqueous extract was added back to the fibers and incubated for 1 hr at 37 °C. Note the disappearance of the carbohydrate at the ten minute point and the appearance of several new carbohydrates.

Those at around 15 minutes are GC-1 compounds and those around 20 minutes are GC-2 compounds. If the aqueous extract is boiled prior to being added back to the fibers, the results are unchanged. If the fibers are boiled prior to the incubation, there is no change in the carbohydrates during the incubation. This clearly shows that the reaction is driven by insoluble enzymes in the fibers. If the fibers are dried and weighed following the incubation a significant increase in weight is detected. This shows that insoluble carbohydrates are added to the fibers (i.e., to the cell walls); the changes seen in the soluble carbohydrates are probably simply incidental to the cell wall additions. Fig. 5 shows a similar experiment carried out using fibers from nonirrigated plants that were undergoing drought stress. The parent application demonstrates that GC-1 and GC-2 compounds are sensitive indicators of stress. Here we see that these differences are also demonstrated by the *in vitro* incubation experiments.

One might ask whether the drought stress differences are due to changes in the insoluble enzymes, changes in the availability of carbohydrate precursors or both. It is well-known that carbohydrates are involved in drought response of stressed plants. For example, glycerol or trehalose may accumulate in response to drought and act as protective agents to maintain cell and membrane integrity. Clearly if carbohydrates are diverted to produce protective materials, the carbohydrate pool available for cell wall synthesis may be altered. The present study has also demonstrated that the carbohydrate patterns fluctuates during the day as the rate of photosynthesis increases (early to mid day) and then decreases (mid to late day). This is demonstrated in Fig. 6 which shows the aqueous extracts taken from fibers harvested at three different times of day (early, within one hour of dawn; mid, within one hour of noon; and late, within one hour of dusk). Of course, the activities of various cell wall enzymes might also vary during the day, thereby further complicating the picture.

The likelihood that stress changes in cell wall carbohydrates is due to carbohydrate pool shifts rather than changes in wall enzymes has also been

demonstrated by combining aqueous extracts of normal fibers with fibers from drought stressed plants and vice versa. In either case the resulting profiles are largely controlled by the source of the extract. That is, extracts from normally grown fibers give essentially normal profiles when incubated with fibers either from normal or stressed plants. Similarly, extracts from stressed plants give abnormal profiles when incubated with fibers from either normal or stressed plants. However, adding known substrates to the normal or stressed fibers gives some indication that the fibers also control the final soluble carbohydrate profiles.

Fig. 7 shows a series supernatants incubated with fibers from irrigated plants. In these experiments a known concentration of substrate (25 mM inositol, 25 mM glycerol, 25 mM sucrose and/or 25 mM arabinose) was incubated with a known weight of fiber. The control contains the normal fiber extract but was boiled to inactivate the enzymes; in the other cases the normal soluble carbohydrates were supplemented with the indicated substrates.. Note that the different substrates produce different patterns—indicating that changes in pool size greatly affects the ultimate carbohydrate pattern. For example, addition of inositol greatly potentiates the consumption of sucrose (large peak near 10 minutes in the control). The addition of glycerol, inositol and arabinose to the sucrose results in enhanced consumption of the sucrose. Fig. 8 shows the same experiment performed with fibers from a drought stressed plant. While similar to Fig. 7, there are clearly differences. The overall level of soluble carbohydrates is lower, but sucrose is clearly consumed. Part of the difference may be that the stressed fibers have a different ratio of enzymes to dry weight than the normal fibers and these experiments were normalized by weight of fiber added. If anything the drought stressed fibers appear to metabolize almost all of the added carbohydrates into insoluble compounds. While this does not preclude there being a different ratio of the various enzymes, it certainly indicates the presence of active enzymes in the stressed material.

Acid Extractable Multimers

Perhaps the most exciting and unexpected discovery of the present research was that following the aqueous extraction it is possible to extract a multimer fraction by boiling for 30 minutes in dilute 0.1M HCl. Figure 9 shows this unique multimer pattern extracted from cotton bolls from 15 to 39 days post anthesis. The older bolls have exactly the same multimers but in a lower per weight amount. Presumably these multimers represent some component that connects the paracrystalline cellulose in the wall. Like the GC-2 compounds the multimers are reducing sugars indicating a nontypical glycan linkage in the polymers (see Fig. 3). Hydrolysis of individual peaks has shown that they contain galactose, glucose and mannose. In classical plant cell wall research dilute mineral acids are sometime used to extract pectins or "pectic materials" which, by definition, contain galacturonic acid residues. Clearly, the multimers are not pectins or pectic materials. Further, it is necessary to first perform the cold aqueous extraction so that the multimers are not obscured by the GC-1 and GC-2 compounds. Fig. 10 shows the HCl multimers extracted from fibers on a drought stressed plant. Clearly the multimer pattern is disrupted particularly at around 15-20 minutes of retention time. The disruption of the multimer patterns is a very sensitive detector of stress and obviates the need for quantitative comparisons as is often required in making GC-1 versus GC-2 based stress detection. Further analysis of the multimers of normal fibers has revealed that the major difference between successive multimers is in addition of glucose units. That is, successive multimers in a series have comparable amounts of galactose and mannose but different amounts of glucose. It is not yet known whether the abnormal multimers of the drought stressed plants follow this pattern. It appears certain that many of these same multimers are found in a variety of cell walls. Figure 11 shows that HCl extracts of sugar beet root tissue contains a multimer series wherein several of the compounds exactly overlap some of the cotton multimers.

The multimer extraction is ideally suited for evaluating cotton fiber samples for a number of defects that plague the textile industry. Motes are immature, short fibers that lower the quality of cotton. Although their presence can be assessed by microscopic inspection of fibers, they also give a unique carbohydrate pattern allowing determination of mote contamination. Of even more importance is the presence of "white speck" fibers which are abnormal fibers that do not take up dye normally. Although this defect can be assessed by dying and inspecting the fibers, analysis of HCl multimers provides a ready way of assessing the presence of white speck fibers. As shown in Fig. 12 and Fig. 13 individual white speck (w) HCl extracts show significantly higher arabinose to glucose ratio than do the extracts (p) of normal fibers.

Interestingly, aqueous extracts of fibers from extremely drought stressed plants show some of the multimers. Preliminary experiments have indicated that these multimers are similar if not identical to those released by the dilute HCl treatment. The real question is why they are released by a simple aqueous treatment. One can hypothesize that the multimers are part of a hemicellulosic "glue" that holds the cell wall cellulose microfibrils together. Under drought stress conditions carbohydrate shortages and/or enzymatic defects prevent the proper assembly of the cell wall components. In such a case the "glue" does not stick properly and is very easily washed out of the walls. As will be demonstrated below, there are proteins associated with at least some of the multimers (producing a special glycoconjugate). These proteins may well be responsible to producing some of the linkages that hold the multimers into the wall. Certainly, the mild HCl extraction would be adequate to partially denature the proteins and negate their purported binding. Fig. 14 shows the multimers extracted from fibers incubated with the substrates shown in Table 1. The number associated with the particular trace relates to the substrates added. The important point is that the addition of certain substrate combinations (note traces 2 and 8, for example) appear to reduce the extraction of multimers. The control (addition of no exogenous substrates) indicates the normal extractability of the multimers.

Presumably certain substrate combination produce a more tightly cross-linked product so that very few multimers can be readily extracted.

Table 1

Trace #	Glycerol	Sucrose	Raffinose	Cellobiose	Inositol
1	50mM	100mM	50mM	50mM	50mM
2	50mM	100mM	100mM	50mM	50mM
3	50mM	100mM	50mM	50mM	50mM
4	50mM	0	50mM	50mM	50mM
5	50mM	0	50mM	100mM	100mM
6	0	0	0	0	0
7	50mM	100mM	50mM	100mM	0
8	0	100mM	50mM	100mM	50mM
9	0	100mM	50mM	100mM	0
10	50mM	100mM	0	100mM	100mM
11	50mM	100mM	100mM	100mM	50mM
12	50mM	100mM	0	0	50mM

Another surprising finding is that multimers can be extracted from finished cotton fabric as well as from carefully harvested fibers as was shown above. Fig. 15 shows multimers extracted by 30 minutes of boiling in 0.1M HCl from an undyed (off white or ivory) cotton towel and from a dyed cotton towel (green). In each case the top trace represents extraction of a new towel and the second (lower) trace shows extraction of a towel that had been laundered one time. Attempts were made to standardize the amount of extracted fabric. Note that the extracted multimers look very similar to those extracted from specially prepared fibers. In this case processing of the fabric has removed all GC-1 and GC-2 compounds so that an aqueous preextraction is

unnecessary—there is no danger that the GC compounds might obscure the multimers. The differences in quality and quantity of multimers extracted is due either to differences in the starting cotton or the textile processing between the two different fabrics. Experiments with "permanent press" treated cotton indicates that such treatments significantly alter the quantity and quality of extracted multimers. Another important discovery is that cotton fabrics are capable of yielding multimers even after prolonged wear and washing. Fig. 16 shows multimers extracted from an old towel and an old pillowcase in the inventors household. These fabrics had been washed dozens of times and still produced similar multimers. Clearly, the multimer analysis can be used to measure wear-related changes in cotton fabrics and to analyze various fabric treatments for their long-term effects on fabric wear. Any treatment that inhibits the release of the multimers will probably extend the lifetime of the fabric. Although a dilute acid wash is the preferred way of extracting multimers for analysis, it has been discovered that prolonged (several days) aqueous extraction at elevated temperatures also releases the multimers. Presumably long exposure to hot water gradually hydrates paracrystalline portions of the cell wall and allows the multimers to be released. This strongly suggests that these materials are gradually released during washing; undoubtedly the loss of these "glue" elements results in a weakening of the fabric. Traditionally it was believed that fabric weakening with age was merely a mechanical effect of wear and washing. These discoveries suggest that washing actually removes a vital binding component from the cotton. Treatments that slow this removal will extend the life of the cotton fabric. Another practical use of multimer extraction is the determination of cotton types used in a given fabric. Extraction of a range of different cotton varieties has shown reproducible multimere differences between some varieties. In particular certain high grade cottons are derived from different cotton species. It might be very beneficial to have a simple test to detect adulteration of these premium cottons with less expensive "ordinary" cottons.

Protein Glue and the Multimers

Fig. 18 shows the flow diagram of an experiment intended to determine what part, if any, protein plays in the cell wall phenomena discussed above. Fibers were hydrated and then boiled to denature any enzymes and kill any microorganisms (toluene was also added to additionally insure sterility). The fibers were then incubated at 37 °C for 72 hrs either with or without proteolytic enzyme (pronase 1mg/ml). At the end of this time the fibers were separated from the supernatant by centrifugation. The supernatant was then passed through a 0.22 μm pore filter to remove any particulate material (this is standard procedure to protect the chromatographic columns). Surprisingly, the supernatants that were not treated with pronase plugged the filters and remained on the filter surface as a gooey material (retentate). The amount of this material depended strongly on the time of day that the source fibers were isolated. The early (7 am) fibers showed a maximum amount of this material; those from noon fibers showed an intermediate amount; while those from the late (7 pm) fibers showed a minimum amount. It is believed that this gooey retentate represents the "glue" that holds the cellulose in the cell wall. Obviously, rates of cell wall synthesis vary with time of day, and the rate of synthesis might affect the extractability of the glue material. If the filtrate (primarily from the pronase-treated samples) is treated with HCl, a typical multimer pattern is generated. Significantly, if the retentate is treated with HCl, or with proteolytic enzyme multimers are generated. This indicates that long-term aqueous extraction removes a cell wall component that includes the multimers. This material is macromolecular and forms a gooey gel. If the material is treated with proteolytic enzyme, the gel is destroyed and the multimers become soluble. The fact that this gel is held together by bonds sensitive to proteolytic enzyme strongly suggests that proteins are important is gluing the cell wall together.

Fig. 19 shows the multimers produced from the HCl-treated retentate of the early (top graph), midday (middle) and late (bottom)fibers. Each graph shows carbohydrates and protein (A_{280}). Note that certain of the multimers are clearly

associated with proteins. Further, the precise nature of the proteins changes with time of day. The early and late graphs show a protein triplet peak between 20 and 25 minutes while the midday graph shows a prominent protein peak at about 35 minutes. As shown in Fig. 20, treatment of the samples with either high purity trypsin or high purity chymotrypsin removes the protein components and causes the joint protein/carbohydrate peaks to either disappear or change in shape or position.

Boiling the cotton fibers prior to glue extraction resulted in a significant difference in the pattern of multimers which was obtained. With fibers collected in the morning (7 am) boiling resulted in the absence of two or three peaks as shown in Fig. 20. However, with fibers from balls collected at noon, boiling resulted in the omission of almost all but the first multimer in the series which was relatively more abundant on a per mg fiber basis. This is shown in Fig. 21. Fibers collected in the evening (7 pm) produced a multimer pattern some what intermediate but more like the noon fiber pattern. These results are consistent with the fact that the majority of cell wall synthesis occurs at night. The morning fibers are the remaining material from wall synthesis, noon fibers represent the lowest point of wall synthesis and the evening fibers are representative of the beginning of the wall synthetic process.

The chromatograms of the multimers are characteristic of having a slight tailing edge of the earlier eluting peaks. This is suggestive of incomplete resolution of peaks. This was investigated by doing a time course of hydrolysis of the glue as shown in Fig. 22. The five-minute time point demonstrates that these early peaks consist of two small peaks which then, with longer hydrolysis times result in one large peak with a tailing edge. This indicates that there are two peaks eluting very near to each other but that one is much more abundant than the other. This apparent incomplete resolution of peaks was also investigated by subjecting the 30-min hydrolysate to filtration in molecular weight cut off filters (MWCO). This result is shown in Fig. 23 in which filters of 30,000, 10.000 and 4.000 MW were employed. The major portions of the multimers were obtained in the 10.000 MW retentate or in the 4,000 MW filtrate.

Cotton fibers (25 DPA) were extracted with water at three temperatures, 37, 25and 4°C for up to 30 days. The extraction tubes were sonicated for 15 min, and filtrates were removed each day and centrifuged to yield a white particulate pellet. It was observed that between 3 and 9 days of extraction the fibers extracted at 4°C were characterized by one obvious difference. Before sonication the tubes all looked alike but following sonication the 4° tubes became turbid indicative of a very fine particulate suspension. Yet on centrifugation, the pellets obtained from all of the tubes were similar in the quantity of precipitate and in the pattern of multimers obtained (Fig. 24). This result is indicative of a temperature dependent and thus presumably enzymatic process which produces larger particles at the warmer temperatures.

White flakes

During the development of the cotton fibers there are some structures present which appear as white flakes in dried material. This material is presumed to result in large part from the drying of the liquid inside the developing carpel; however, the flakes may also be apparent in freshly opened bolls. The flakes are obvious up until at least 39 DPA in many instances, but they disappear in the later stages of development and are gone by the time the bolls open at maturity. Although many investigators have mentioned the white flakes informally, there does not appear to be any investigation of them in the literature. I have dissected the white flakes from the fibers and looked at both independently for the soluble oligosaccharides. I have done the dilute acid extraction to obtain the multimers. On a dry weight basis, the white flakes release at least 5-10 times the quantity of multimers as do the fibers. This is shown in Fig. 25. Since these white flakes contain multimers which eventually end up in the fibers, the obvious conclusion is that the white flakes contain precursors to the fiber wall. Therefore, all of the developing fiber wall material does not originate within that particular fiber. At this time I do not know if the white flakes originate from a particular population of fibers, other cells in the developing seed coat or other tissues from the inner carpel wall.

Tunicamycin experiment

The effect of tunicamycin on the multimers obtained from cotton fibers was investigated. Fibers were incubated with water for two days to deplete endogenous substrates and then incubated for another 24 hours with or without tunicamycin (10μg/ml) both with and without added substrates. Tunicamycin specifically inhibits the formation of the bond between asparagine and N-acetylglucosamine in N-linked glycoproteins. Without added substrates, the tunicamycin had no appreciable effect but with added substrates such as cellobiose and raffinose, the effect was dramatic as the tunicamycin inhibited the quantity and patter of the multimers extracted as shown in Fig 26. In other experiments, not shown here, the tunicamycin effect was variable if the endogenous substrates were not depleted prior to addition of tunicamycin.

Enzyme Treatments

Extracted multimers were subjected to incubation with a cellulase (*Trichoderma reesei*) or a β-glucosidase (almond emulsin). The effect of the β-glucosidase appeared to be to increase the heights of the multimermer peaks significantly and to generate one additional small peak with a retention time slightly greater than 20 minutes. Presumably this is the result of removing terminal glucose unit(s) which results in a compound with an increased detector response. The cellulase gave a very different result since it resulted in the near elimination of many peaks and great reductions in many peak heights with a great increase in the peak height of the first peak in the series of multimers as shown in Fig. 27. The cellulase result, with the exception of the peak at 11 min (related to cellobiose), was very similar to the profile of the fibers from the stunted plant.

Based on the results of the treatment of the isolated multimers with enzymes, it was decided to attempt to modify the multimers in situ by subjecting the fibers to a sequential enzyme treatment. The goal was to be able to specifically remove the multimers by the chemically gentle and specific enzymatic means. If this could be

accomplished then one could make a cogent argument for the multimers as specific components of the fiber cell wall. Fibers (25 DPA) were subjected to a 24 hr incubation with trypsin, chymotrypsin, proteinase K or pepsin followed by a second 24 hr incubation at 37°C with cellulase or β-glucosidase. Alternatively, a duplicate set of samples was subjected to the same enzymes but in the reverse order. That is the cellulase or β-glucosidase first and then the protease second. The final fiber/residual material was then subjected to the dilute acid extraction to remove the multimers prior to HPAEC-PAD.

As shown in Figs. 28-30, material was released by both proteases and cellulase or β-glucosidase. The multimers extracted from the final released material (Fig. 30) indicate that multimers could be extracted from the control fibers or fibers subjected to protease first followed by cellulase, but no multimers were obtained from the material subjected to the cellulase first followed by the protease. In that case chymotrypsin was the most effective protease just as it was for degradation of the glue. However, the most striking observation was that the fibers treated with cellulase followed by protease lost their structural integrity and simply fell apart or were sucked up into the Pasteur pipette when the extract was removed.

When mature fibers from opened bolls were subjected to the same cellulase followed by protease procedure, very little happened so the procedure was repeated a second time. At the end of the second cycle, the fibers completely lost their structural integrity and only a precipitate of very small particles remained. These particles were then washed, subjected to digestion either in dilute HCl, in 2N trifluoroacetic acid or in 6N HCl. Actual digestion occurred only in 6 N HCl, and the resulting monosaccharides obtained appear to be in excess of 99% glucose. This indicates that sequential treatment with cellulase followed by protease is an excellent method for producing cellulose of extremely high purity.

This result is striking since it provides evidence for significant modification of the fiber walls associated with boll opening and maturity. This means that even though the cellulosic fiber wall is deposited in daily growth rings, there is obviously a very significant post-depositional modification process which drastically alters the fiber wall properties.

Protein Glue and Crosslinking

The probability that cellulose microfibrils of the plant cell wall are embedded in a matrix that "glues" them together has been proposed by a number of investigators over the years. The nature of such a glue matrix has been the subject of considerable discussion but there has been no characterization of such a matrix material. The presence of cell wall subunits, in cotton fibers, was proposed by W. Lawrence Balls (Balls, W. Lawrence, 1928, *Studies of Quality in Cotton*, Macmillan & Co., London.). The present work (see above) on the cell wall "glue" matrix is an extension of work in my laboratory to characterize soluble oligosaccharides and the sucrosyl oligosaccharides in particular which appear to be involved in developmental changes of the cotton fiber.

Mature fibers from opened bolls were extracted with cold water and the extract was removed. Cross-linking was then accomplished using water soluble carbodiimide in unbuffered water. The pH of the reaction mixture was measured and determined to be between 5.0 and 5.2. Two concentrations of water soluble carbodiimide were used, 125 mM and 250 mM. The crosslinking reaction was carried out for 2 hr at room temperature followed by overnight at 4°C. The reaction mixture was washed from the fibers and the enzymatic digestion then ensued. The fibers were incubated with cellulase (*T. reesei*)(1mg/ml) for 24 hrs followed by chymotrypsin (CT) (1mg/ml) and the incubation sequence was then repeated. The results are shown in Figures 31-34. In all cases, sample number 1 is the control; no 2 are the fibers from the 125 mM carbodiimide reaction and number 3 are the fibers from 250 mM carbodiimide

reaction. Under the reaction conditions the carbodiimide would be expected to promote amide bond formation between amino acids .while having negligible effect on ester bond formation between carbohydrates.

I have been able to extract a series of oligomers (multimers) from developing cotton fibers by both chemical and enzymatic methods. These multimers have PAD retention times of 14 minutes and greater under the conditions analyzed. The regular spacing of the peaks is indicative of a series of oligosaccharides varying by a unit monomer in size. These multimers are heteropolymers with a repeating glucan unit extending from a core peptidoglycan structure.

Prior work in my laboratory has shown that 25 DPA cotton fibers can be degraded by a sequential enzymatic treatment with a cellulase followed by a protease but the reverse sequence does not achieve the same result. The fibers completely lose their structural integrity. When fibers from bolls that have opened are subjected to the same sequence they do not lose their integrity unless the process is repeated a second time. Following the second protease treatment, there is a white particulate precipitate in the bottom of the tube.

Quantitatively the constituents released by enzymatic treatments consist mainly of glucose (Glc) and cellobiose (CB). Carbohydrates released by the first cellulase treatment is shown in Figure 31 which demonstrates that both carbodiimide concentrations dramatically reduced the amount of glucose or cellobiose released by the cellulase treatment. The peak at 3.5min retention time is arabinose. Many more of the peaks in the 14-20 min range are released by the cellulase from the control fibers than from the treated fibers. It is very significant to note that the major peak with a retention time of approximately 14.5min released from the control fibers has a distinctly shorter retention time than the major peak at about 14.65 min released from the treated fibers. This is a significant difference and it only is demonstrable in the first cellulase extract.

The carbohydrates released by the first chymotrypsin treatment are shown in Figure 32. More peaks in the 14-20 min range are released from the control fibers than the treated fibers in addition to the large amounts of glucose and cellobiose released from both treated and controls fibers. This pattern is consistent for the carbohydrates released by the "second" cellulase treatment (actually a cellulase treatment following a chymotrypsin treatment) (Figure 33) and "second" chymotrypsin treatment (actually a chymotrypsin treatment following a cellulase treatment)(Figure 34).

The carbohydrate peaks released with retention times between 14 and 20 minutes also contain a constituent, which absorbs at 280 nm as shown in Figure 35. The absorbance at 280 nm is usually due to the phenolic amino acids, phenylalanine and tyrosine in proteins although other compounds may also absorb at 280 nm. Based on this result along with the material released by the proteases, it is concluded that the carbohydrate peaks in this 14-20 min range are glycoproteins. The fact that linking with a carbodiimide renders these carbohydrates more resistant to the protease release further substantiates the conclusion that they are, in fact, glycopeptides. The fact that the protease digestion significantly increases the release of glucose and cellobiose confirms that the cellulosic constituents of the wall are crosslinked by a protease sensitive component (i.e., a protein or glycoprotein).

The white rod-like particles released by the enzymatic degradation of the fibers were subjected to further purification. They were treated with 0.1N HCl for 30 min in a boiling water bath which failed to solubilize them. The residue was also not soluble in 2N trichloroacetic acid at 100°C for 2 hr but was completely dissolved by treatment with 6N HCl at 100°C for 2 hr. The 6N HCl hydrolyzate was then chromatographed under conditions, of 15 mM NaOH, which resolves monosaccharides. The result is shown in Figure 36, which shows a single peak with a retention time identical to that of glucose. It is not possible at this time to determine if the unstable baseline before the glucose peak and after it is significant. The dip at approximately 15 min is due to dissolved oxygen and is a well-known phenomenon. This matter must be further

investigated to determine if there is any significance to these regions of unstable baseline—e.g., whether additional minor carbohydrate constituents are present. At this time it appears that the white particles are essentially pure cellulose and yield only glucose upon hydrolysis in 6N HCl.

As detailed above, I have been able to obtain the oligomers (multimers) from a large molecular complex which is secreted by fibers, *in vitro*, by a temperature dependent mechanism. The relative distribution of the multimers can vary depending on the exogenous substrates incubated with the fibers and the time of day which the bolls were collected. Under optimal conditions I have been able to demonstrate the presence of the multimers in an initial soluble fraction, a secreted fraction which will not pass through a 0.2 μm filter, the precipitate of the aqueous extract and the fibers themselves. The multimers appear to play a structural role in the integrity of the cotton fiber since recent experiments to extract the multimers using specific enzymes have resulted in a striking loss of the physical integrity of cotton fibers.

The experiments just described demonstrate the A_{280} profile of the material released by the sequential treatment of mature cotton fibers with cellulase, chymotrypsin, cellulase and then chymotrypsin again. These profiles indicates that the multimers are attached to protein. When the fibers are treated with a water-soluble carbodiimide to form amide bonds between the carboxyl and amino groups of the amino acid constituents, the fibers become more resistant to enzymatic degradation. This result shows that bifunctional reagents have applications in the textiles and lead to ways to improve the quality (e.g., durability) of cotton fabric. Above I have shown that normal cotton textiles continually shed water soluble multimers over the life of the fabric. This suggests that fabric wear is at least partially due to loss of soluble material during washing. Chemical crosslinking is a way to reduce this loss and, thereby, extend the life of cotton fabrics. Although this test employed carbodiimide any of a large number of bifunctional reagents known to react with amino groups could be used. These reagents are well known to a person of ordinary skill in the art of protein

chemistry. The significant point is that my experiment is the first demonstration that protein crosslinking reagents are useful to alter properties of cotton and other plant-based textiles.

Hydrolysis experiments on the white particulate material which remains following the enzymatic digestion of the fibers is consistent with these particles being perhaps very highly crystalline cellulose. This result is consistent with the prediction by Balls (1928) that the fiber wall is made up of little domino or brick-like structures which are held together and permit the fiber to be flexible. It is probable that the material which holds the "bricks" together is the "glue" matrix described in part here with the multimers attached to a protein backbone. This result is consistent with the fact that plant breeders directly select for varieties with different fiber properties including strength. It is likely that a matrix protein is a primary gene product while a polysaccharide, such as cellulose, is the product of a number of genes. Thus, direct selection and manipulation by genetic engineering should be more successful on the matrix protein than on the complex of enzymes needed to synthesize cellulose.

In addition to the equivalents of the claimed elements, obvious substitutions known to one with ordinary skill in the art are defined to be within the scope of the defined elements. The claims are thus to be understood to include what is specifically illustrated and described above, what is conceptually equivalent, what can be obviously substituted and also what essentially incorporates the essential idea of the invention. Those skilled in the art will appreciate that various adaptations and modifications of the just-described preferred embodiment can be configured without departing from the scope and spirit of the invention. The illustrated embodiment has been set forth only for the purposes of example and that should not be taken as limiting the invention. Therefore, it is to be understood that, within the scope of the appended claims, the invention may be practiced other than as specifically described herein.

CLAIMS

I Claim:

1. A method for monitoring growth stress in plant material comprising:

freezing and lyophilizing the plant material;

making a cold aqueous extract of the plant material;

re-extracting the previously extracted plant materials with dilute boiling hydrochloric acid; and

analyzing the hydrochloric acid extract to reveal a series of carbohydrate multimers, the pattern of multimers revealing the presence or absence of growth stress.

2. A method of analyzing textiles to predict the effect of different manufacturing steps on textile quality, the method comprising:

extracting various textile samples with dilute hot acid;

analyzing the extract to reveal a series of carbohydrate multimers; and

determining which manufacturing steps alter the pattern of extracted multimers.

3. A method of analyzing cotton textiles to predict the presence of abnormal cotton fibers that have abnormal dyeing properties, the method comprising:

extracting cotton textile samples with dilute hot acid;

analyzing the extracts to reveal a series of carbohydrate multimers; and

determining which extracts contain a higher ratio of arabinose to glucose, such high ratio being predictive of abnormal dyeing properties.

4. A method of altering cotton textiles to reduce extractability of carbohydrates comprising treating cotton fibers with a chemical reagent forms covalent bonds with amino groups present on proteins within the fibers.

5. The method of Claim 4, wherein the chemical reagent is a carbodiimide.

6. The method of Claim 4, wherein the chemical reagent forms amide bonds.

7. A method of enzymatically degrading cotton fibers to yield essentially pure cellulose comprising the steps of sequentially treating the fibers first with cellulase and then with protease.

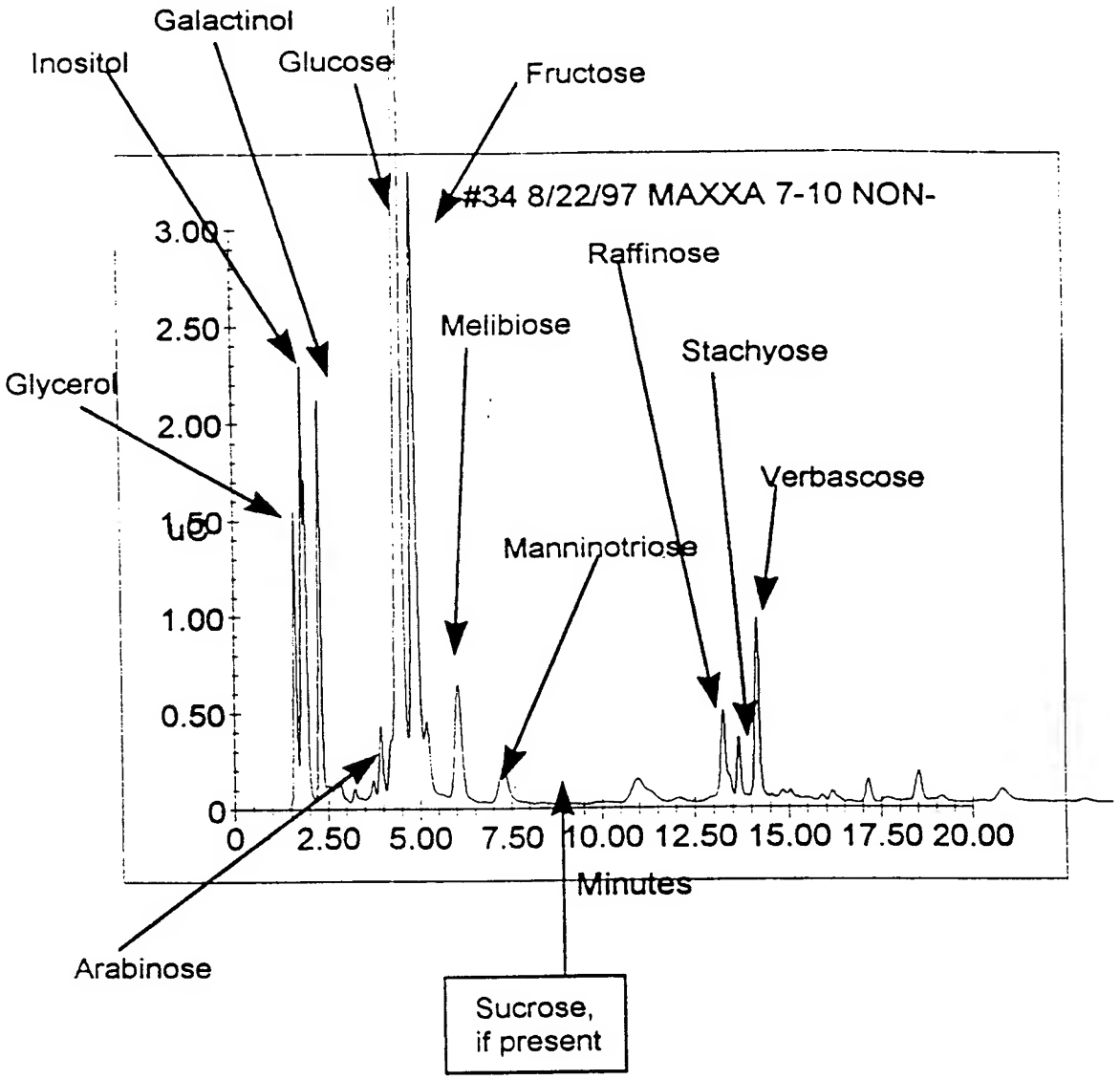

Galactinol
Inositol
Glucose
Fructose
#34 8/22/97 MAXXA 7-10 NON-
Raffinose
Melibiose
Stachyose
Glycerol
Verbascose
Manninotriose
3.00
2.50
2.00
1.50
1.00
0.50
0
0 2.50 5.00 7.50 10.00 12.50 15.00 17.50 20.00
Minutes
Arabinose
Sucrose, if present

Fig. 1

Fig. 2

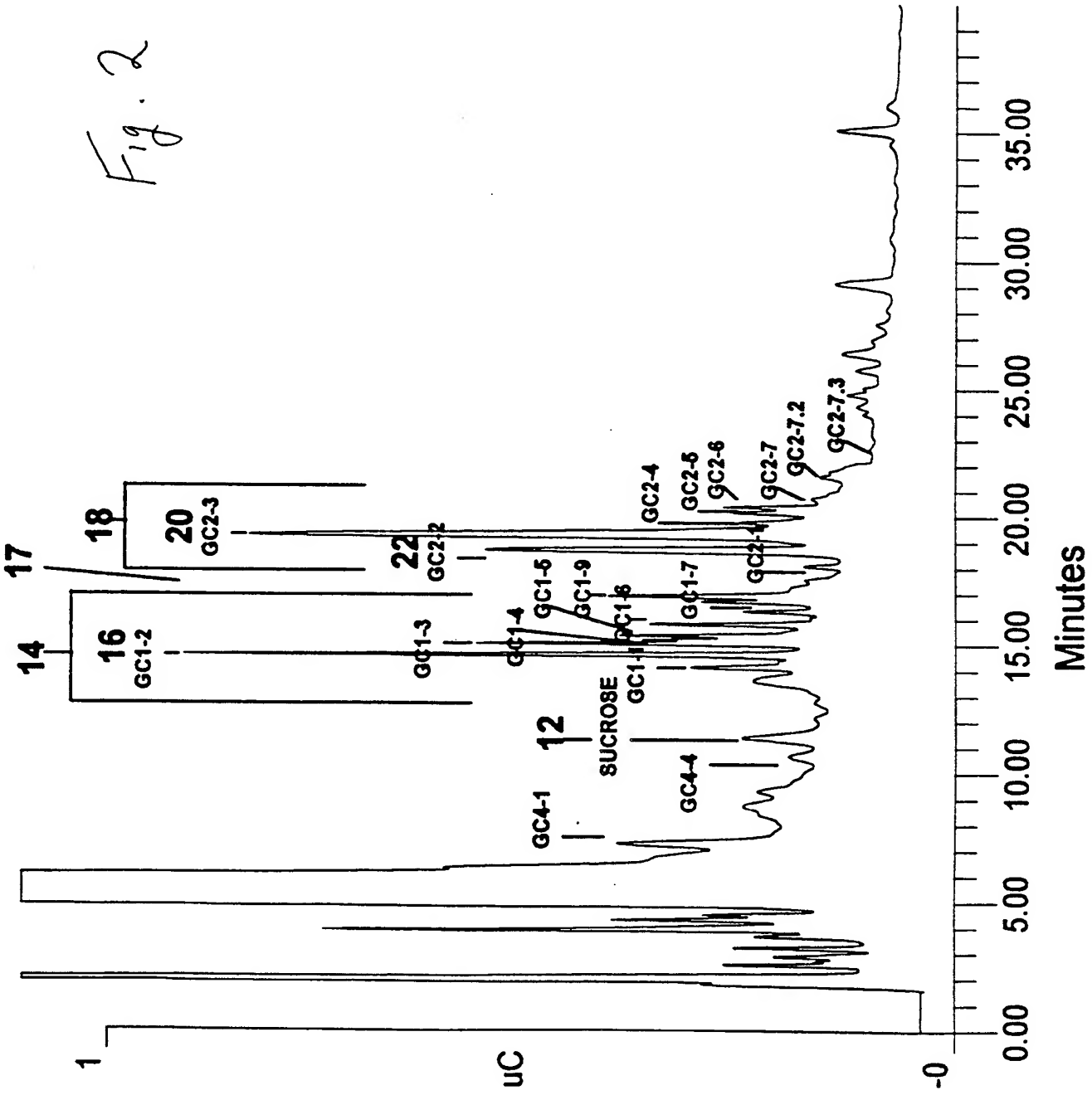
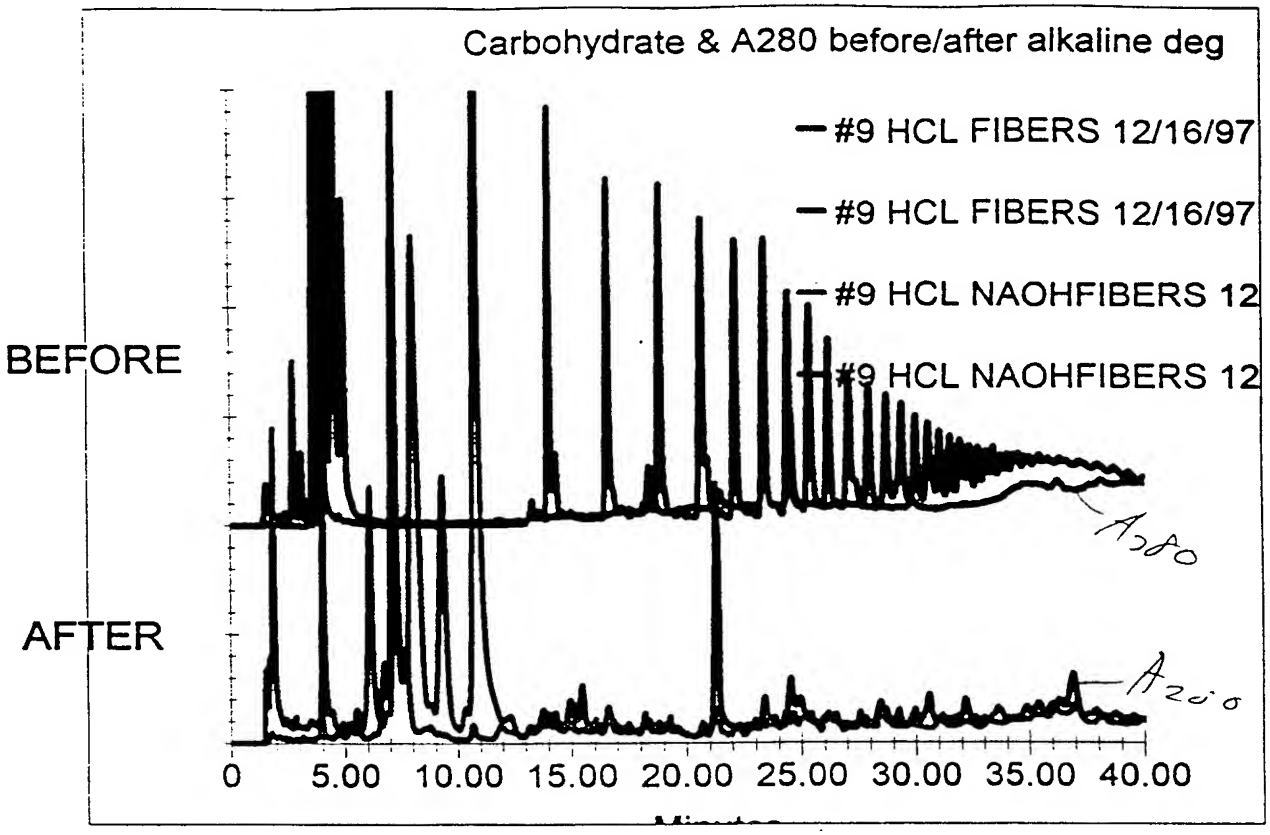

1
uC
-0
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
Minutes
14
16
GC1-2
17
18
20
GC2-3
22
GC2-2
GC1-3
GC1-4
GC1-5
GC1-9
GC1-6
GC1-1
GC1-7
SUCROSE
12
GC4-1
GC4-4
GC2-4
GC2-5
GC2-6
GC2-7
GC2-7.2
GC2-7.3

Fig 3

ALKALINE DEGRADATION



Before and after boiling with 0.1N NaOH for 10 min.

Fig 4

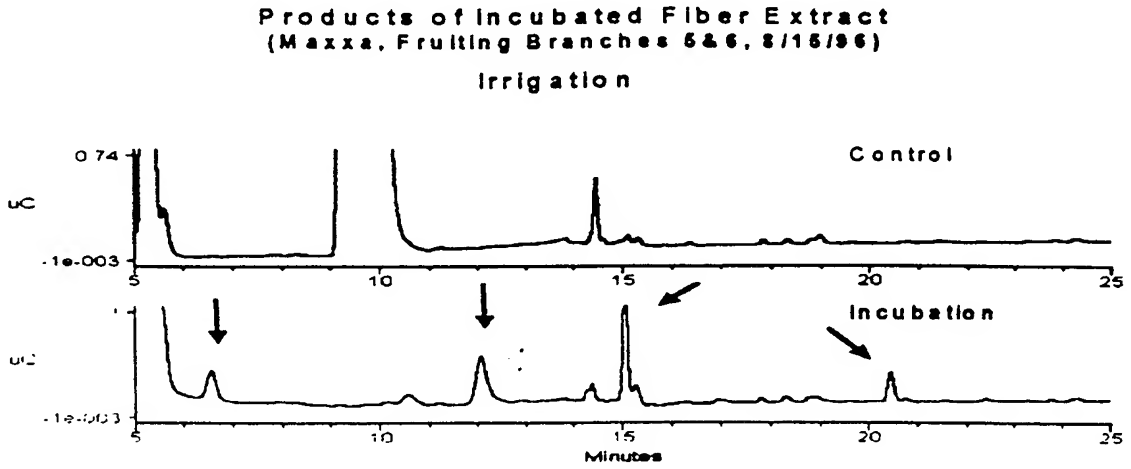
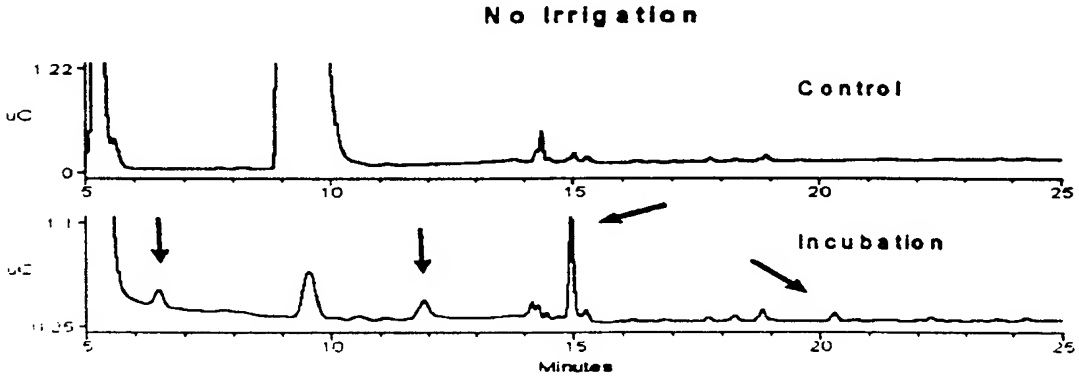

Products of Incubated Fiber Extract
(Maxxa, Fruiting Branches 5&6, 8/15/96)
Irrigation
Control
Incubation
uC
0.74
-1e-003
5
10
15
20
25
Minutes

Fig 5


No Irrigation
Control
Incubation
uC
1.22
0
5
10
15
20
25
Minutes

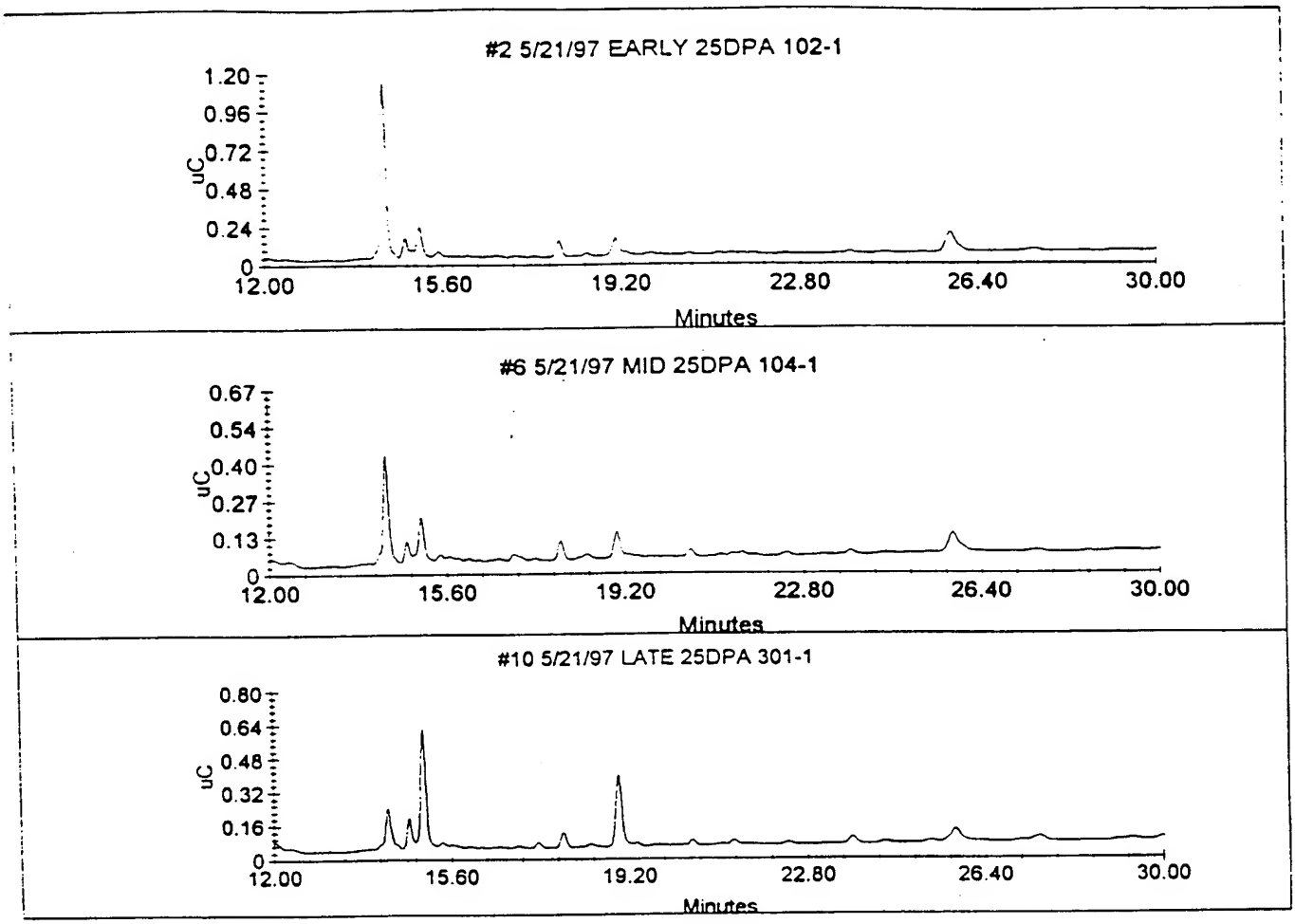

A
#2 5/21/97 EARLY 25DPA 102-1
uC
1.20
0.96
0.72
0.48
0.24
0
12.00
15.60
19.20
22.80
26.40
30.00
Minutes
B
#6 5/21/97 MID 25DPA 104-1
uC
0.67
0.54
0.40
0.27
0.13
0
12.00
15.60
19.20
22.80
26.40
30.00
Minutes
C
#10 5/21/97 LATE 25DPA 301-1
uC
0.80
0.64
0.48
0.32
0.16
0
12.00
15.60
19.20
22.80
26.40
30.00
Minutes

Fig. 6

5 of 32

Fig 7

Irrigation vs. non-Irrigation

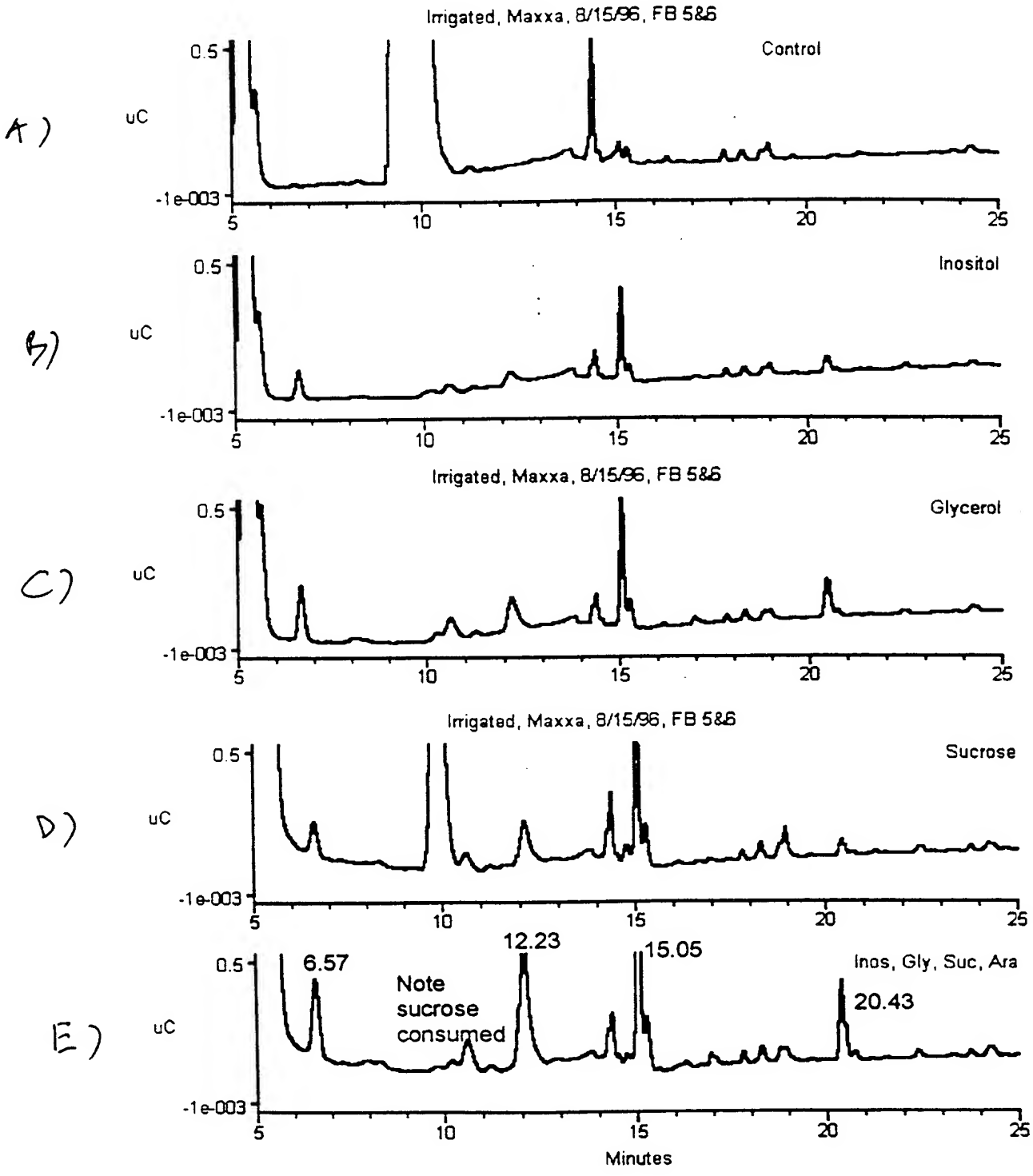
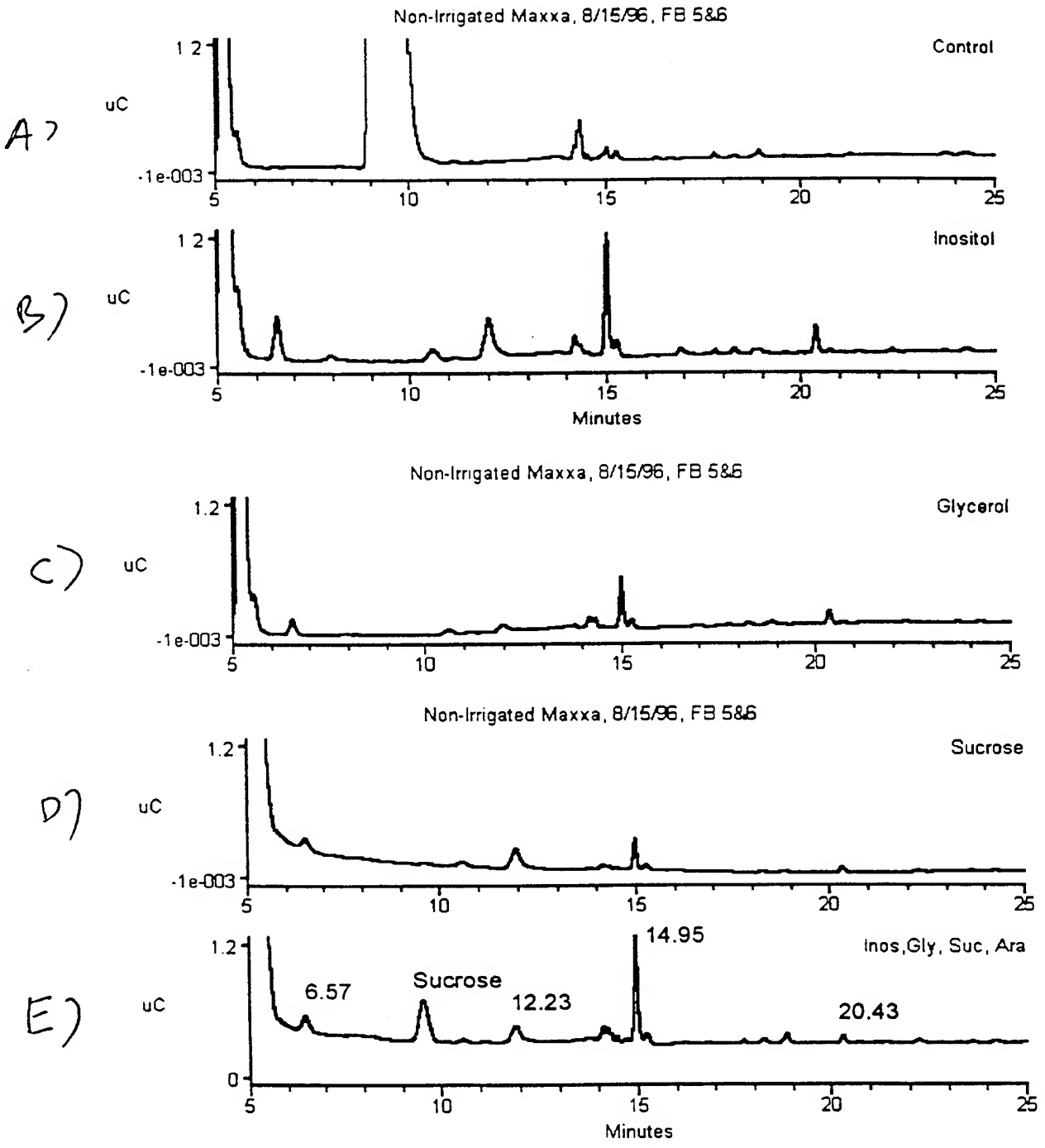


Fig 8


Non-Irrigated Maxxa, 8/15/96, FB 5&6
Control
A)
uC
1 2
-1e-003
5
10
15
20
25
Inositol
B)
Minutes
Non-Irrigated Maxxa, 8/15/96, FB 5&6
Glycerol
C)
1.2
Non-Irrigated Maxxa, 8/15/96, FB 5&6
Sucrose
D)
Inos,Gly, Suc, Ara
E)
14.95
6.57
Sucrose
12.23
20.43
0

Extracted ~Mers from Normal Plant

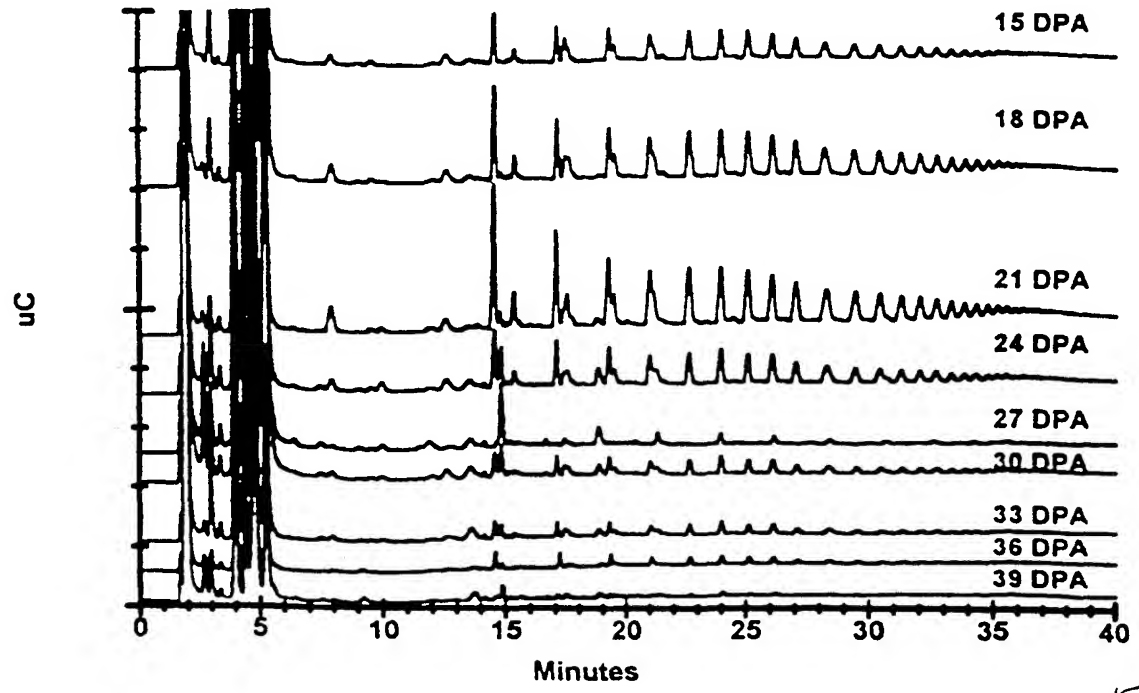


Fig 9

Extracted ~Mers from Stunted Plant

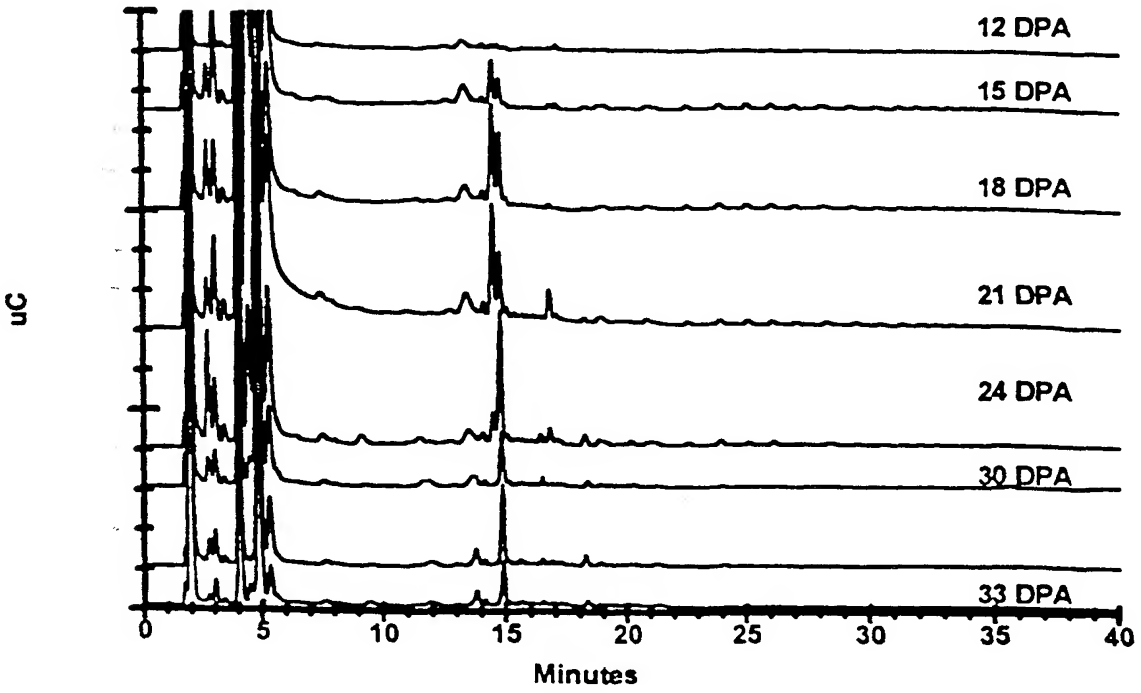
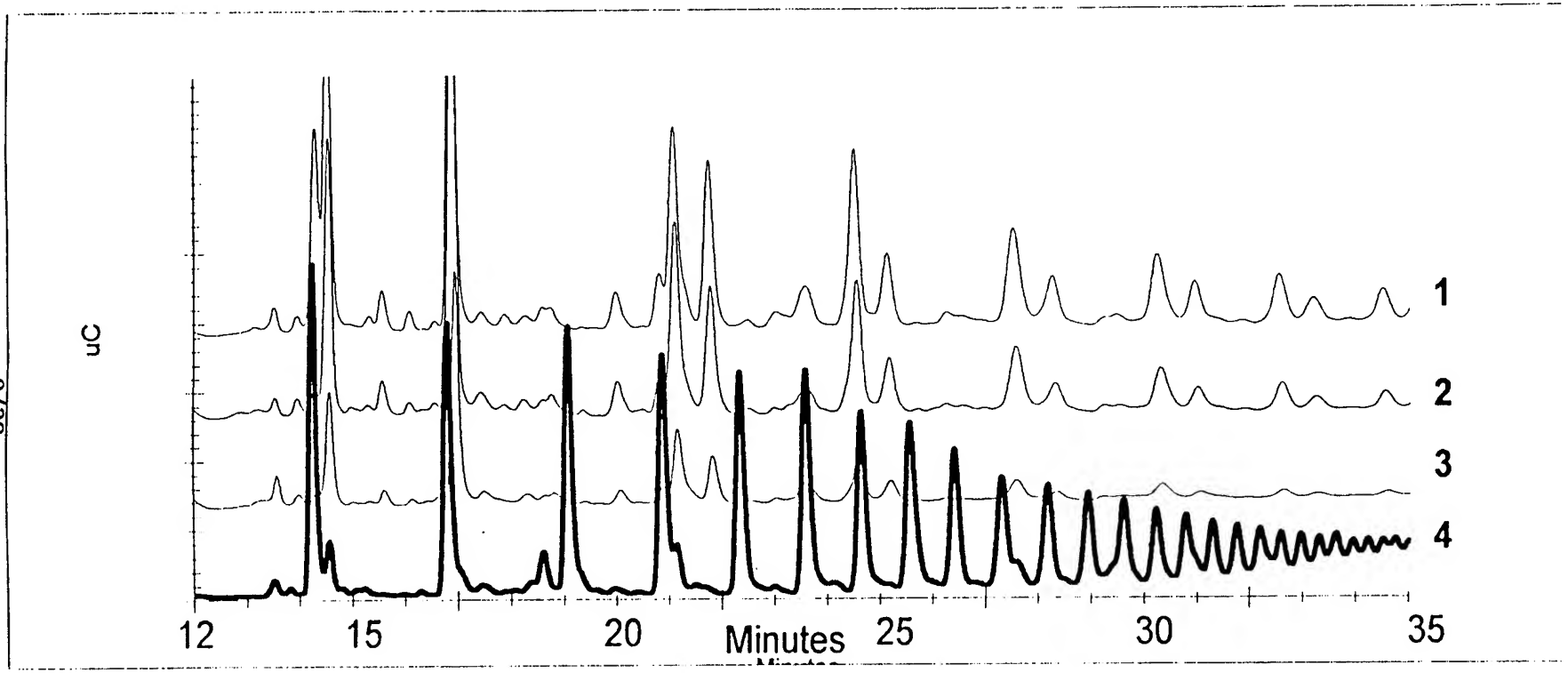


Fig 10

~Mers Extracted fromSugar Beet and Cotton Fiber



Legend:

1. Tap root surface
2. Subdermal
3. Root interior
4. Cotton fiber, 25DPA

Sugar beets from disease category "0"
(no staining, to slight staining within vascular traces from lateral roots - nitroso staining reaction

Beginning of infection process with *Rhizoctonia)*

Fig. 11

Fig 12

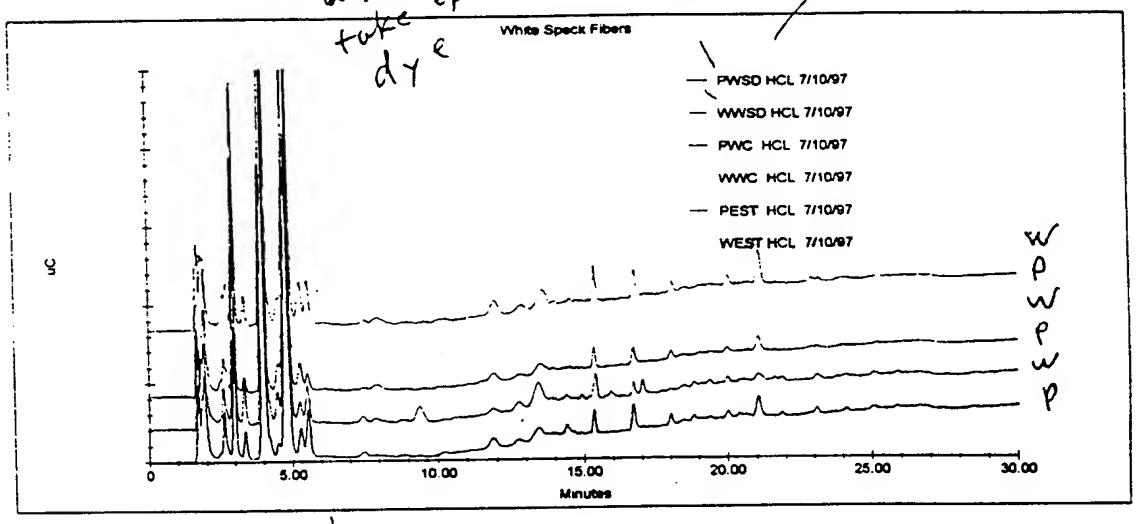

White Speck Fibers
PWSD HCL 7/10/97
WWSD HCL 7/10/97
PWC HCL 7/10/97
WWC HCL 7/10/97
PEST HCL 7/10/97
WEST HCL 7/10/97
0
5.00
10.00
15.00
20.00
25.00
30.00
Minutes

Fig 13

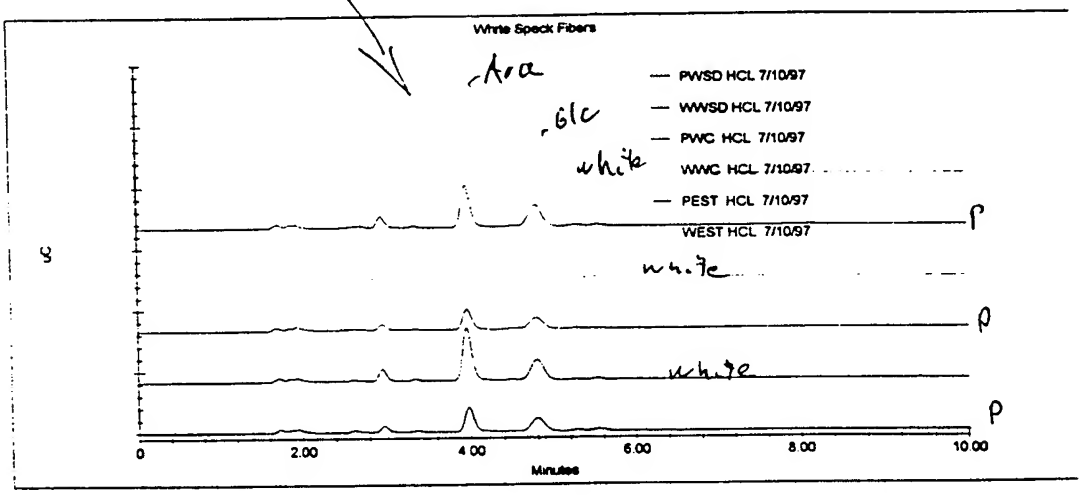

White Speck Fibers
PWSD HCL 7/10/97
WWSD HCL 7/10/97
PWC HCL 7/10/97
WWC HCL 7/10/97
PEST HCL 7/10/97
WEST HCL 7/10/97
0
2.00
4.00
6.00
8.00
10.00
Minutes

Fig. 14

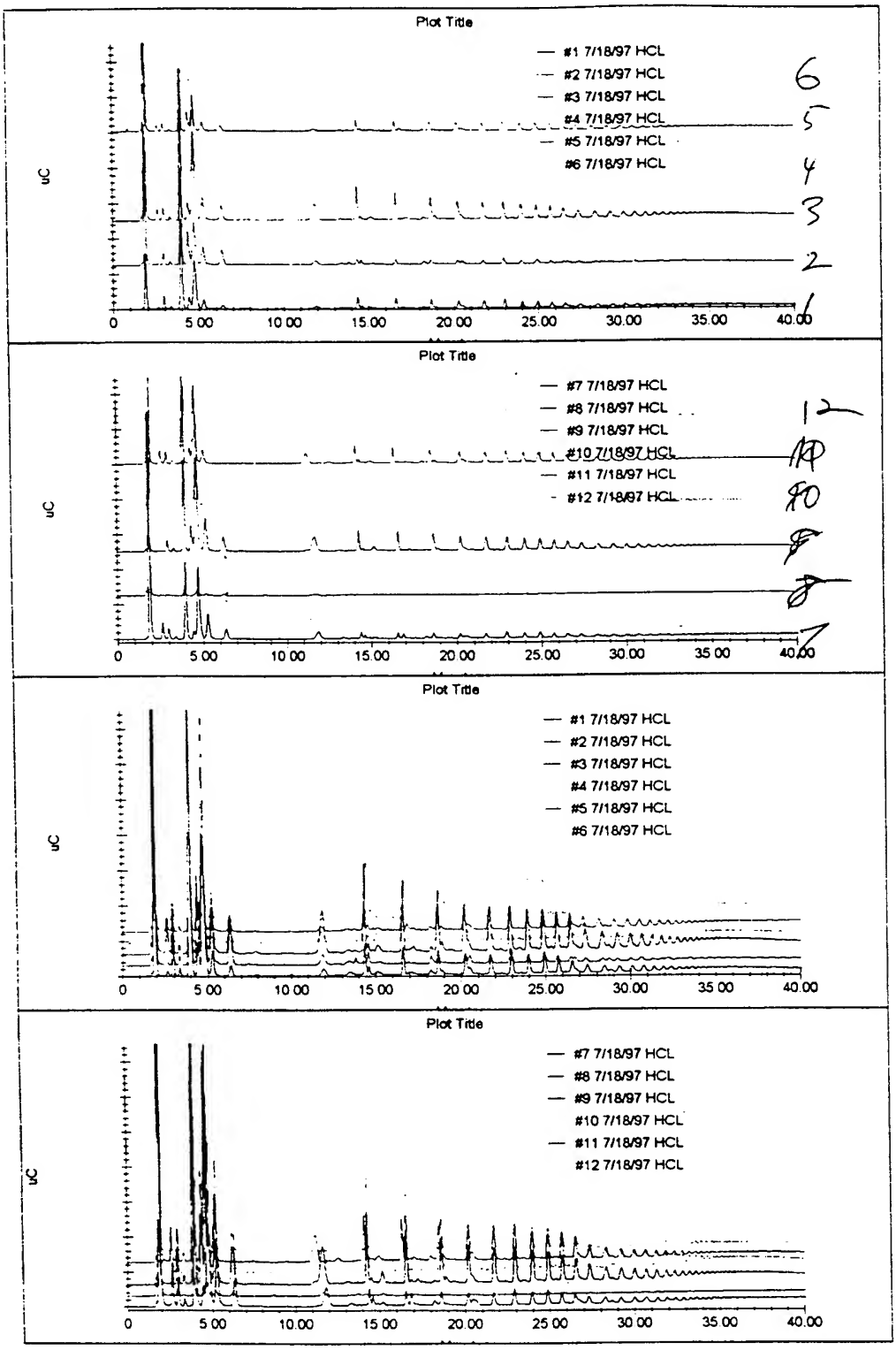
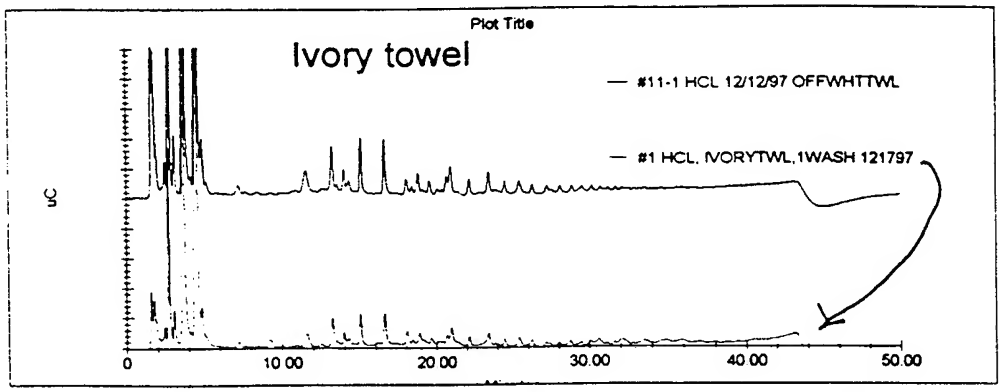
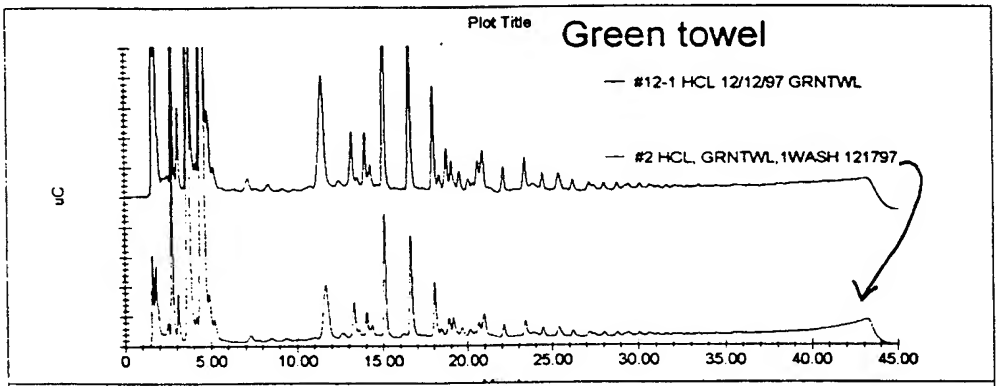

Plot Title
#1 7/18/97 HCL
#2 7/18/97 HCL
#3 7/18/97 HCL
#4 7/18/97 HCL
#5 7/18/97 HCL
#6 7/18/97 HCL
uC
0
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
Plot Title
#7 7/18/97 HCL
#8 7/18/97 HCL
#9 7/18/97 HCL
#10 7/18/97 HCL
#11 7/18/97 HCL
#12 7/18/97 HCL
uC
Plot Title
#1 7/18/97 HCL
#2 7/18/97 HCL
#3 7/18/97 HCL
#4 7/18/97 HCL
#5 7/18/97 HCL
#6 7/18/97 HCL
uC
Plot Title
#7 7/18/97 HCL
#8 7/18/97 HCL
#9 7/18/97 HCL
#10 7/18/97 HCL
#11 7/18/97 HCL
#12 7/18/97 HCL
uC

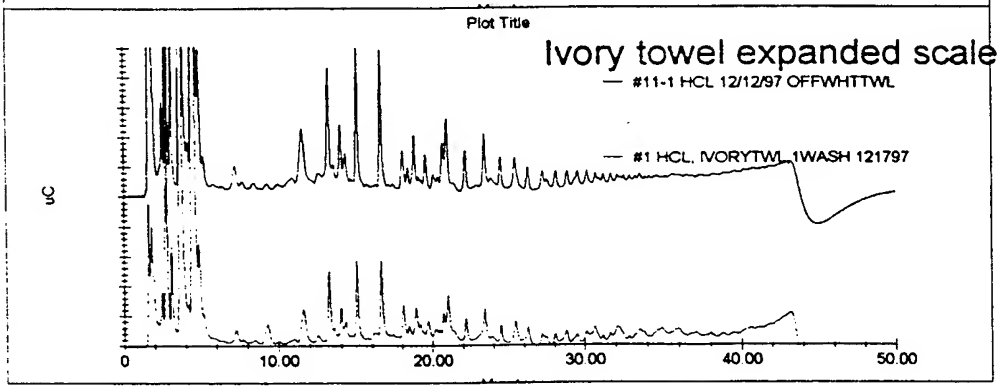
Fig 15

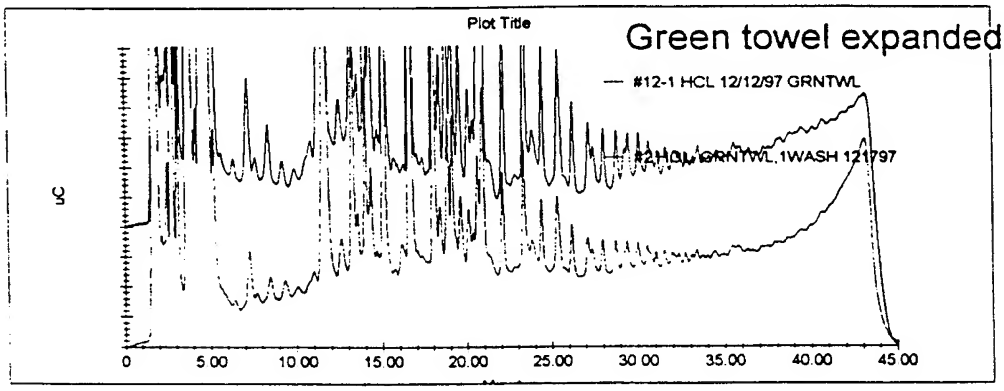
A)


Plot Title
Ivory towel
#11-1 HCL 12/12/97 OFFWHTTWL
#1 HCL, IVORYTWL,1WASH 121797
uC
0
10.00
20.00
30.00
40.00
50.00

B)


Plot Title
Green towel
#12-1 HCL 12/12/97 GRNTWL
#2 HCL, GRNTWL,1WASH 121797
uC
0
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00


Plot Title
Ivory towel expanded scale
#11-1 HCL 12/12/97 OFFWHTTWL
#1 HCL, IVORYTWL,1WASH 121797
uC
0
10.00
20.00
30.00
40.00
50.00


Plot Title
Green towel expanded scale
#12-1 HCL 12/12/97 GRNTWL
#2 HCL, GRNTWL,1WASH 121797
uC
0
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00

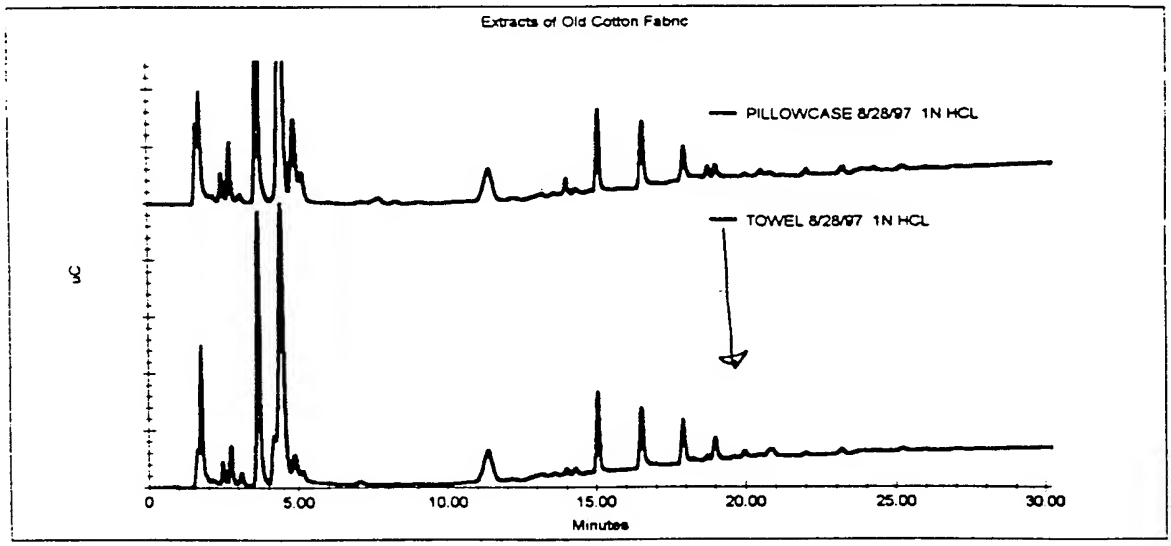
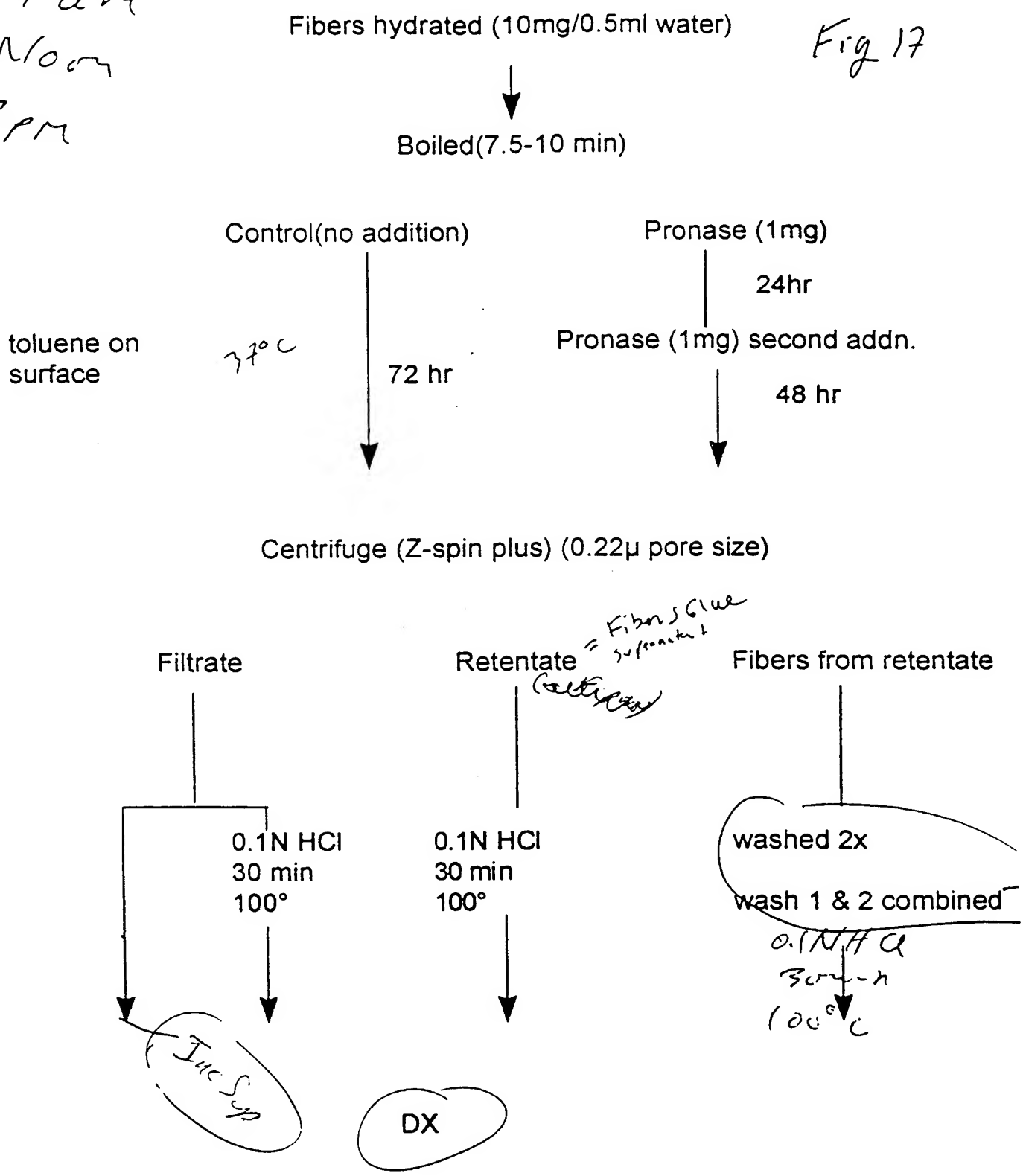

Extracts of Old Cotton Fabric
PILLOWCASE 8/28/97 1N HCL
TOWEL 8/28/97 1N HCL
uC
0
5.00
10.00
15.00
20.00
25.00
30.00
Minutes

Fig. 16

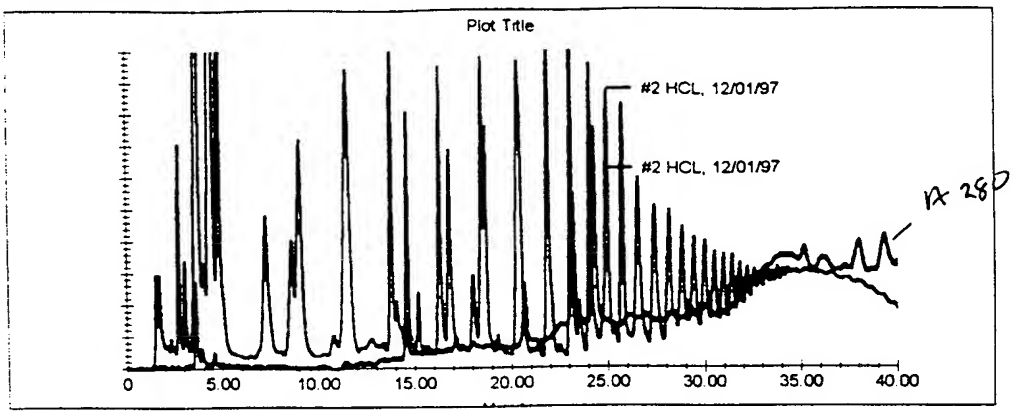
7 am
Noon
7 pm

Fig 17



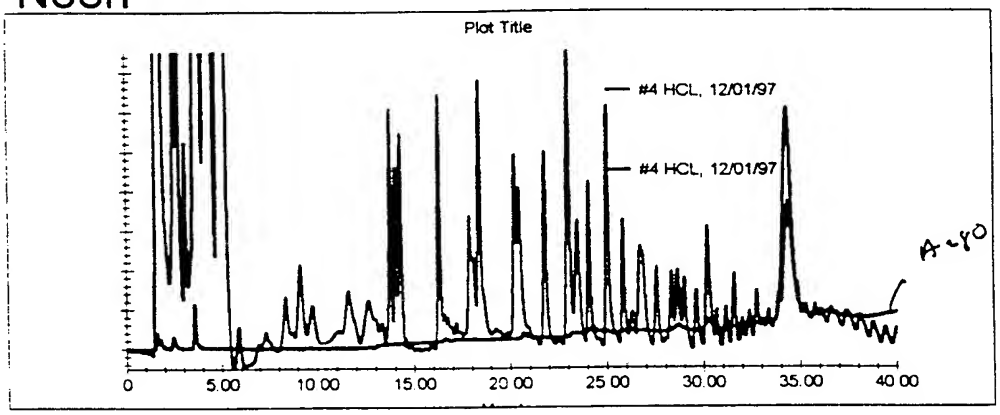
A)

7am fibers



B)

Noon



C)

7 pm

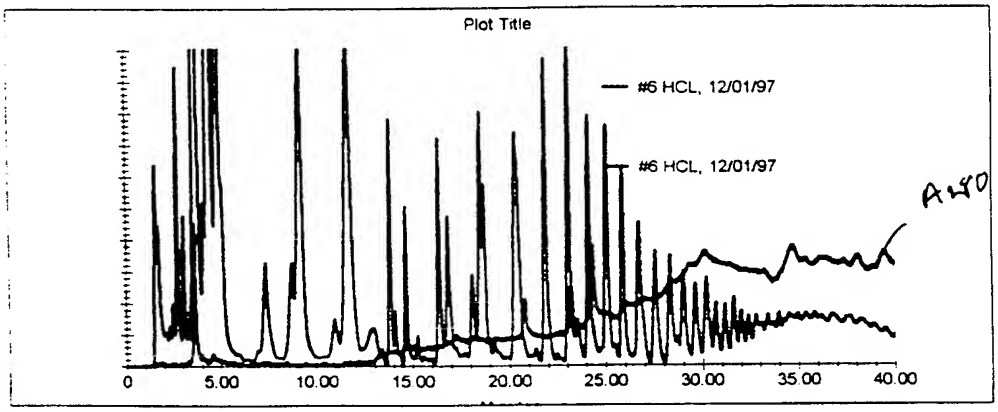
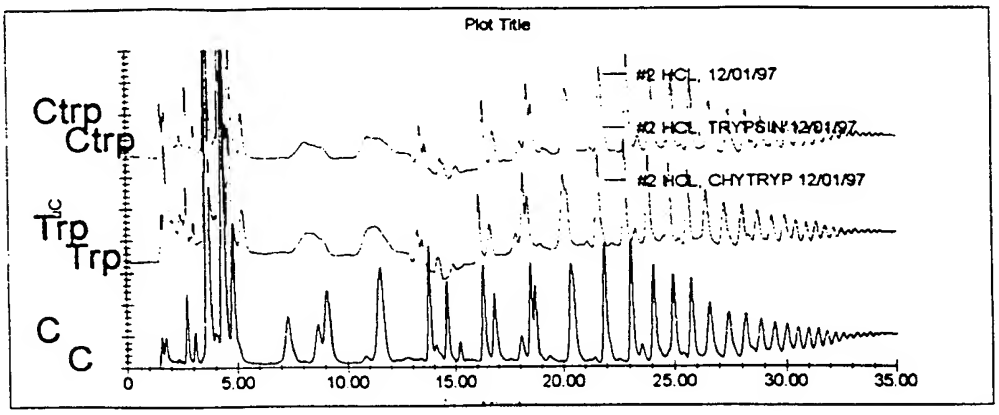


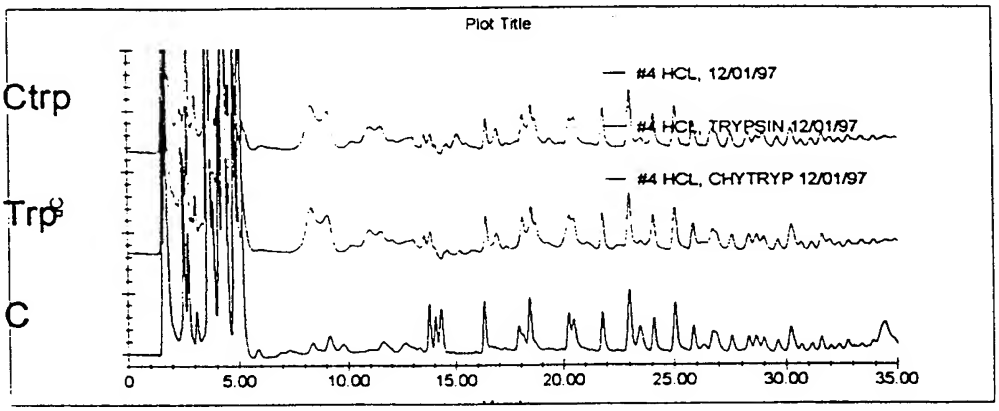
Fig. 18

7 am fibers



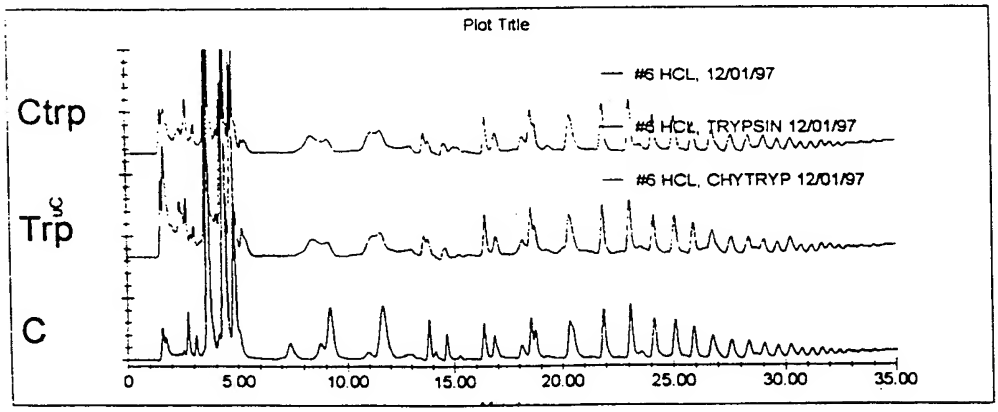
A)

Noon fibers



B)

7pm fibers



C)

Fig 19

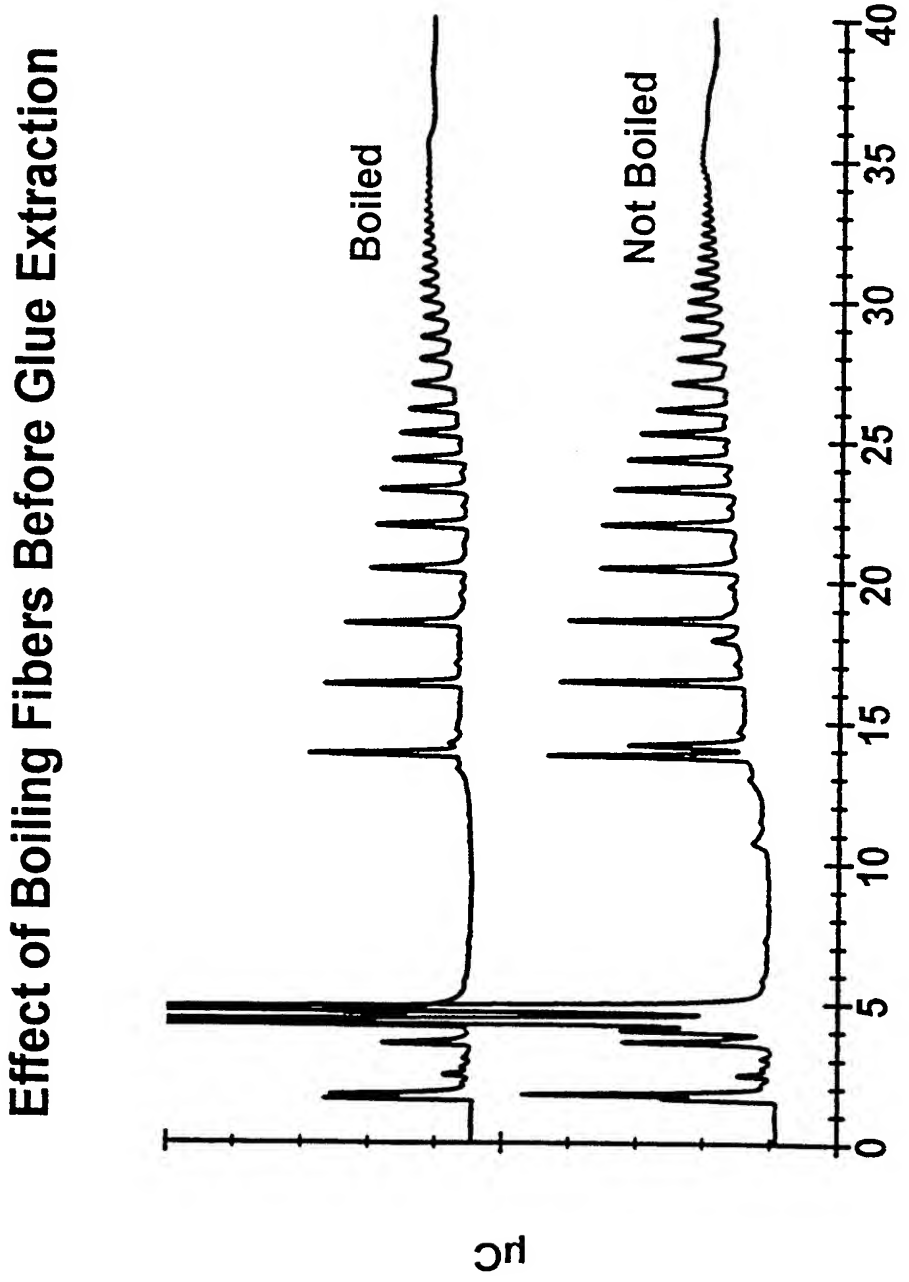

Effect of Boiling Fibers Before Glue Extraction
Boiled
Not Boiled
0
5
10
15
20
25
30
35
40
Minutes
µC

Fig 20

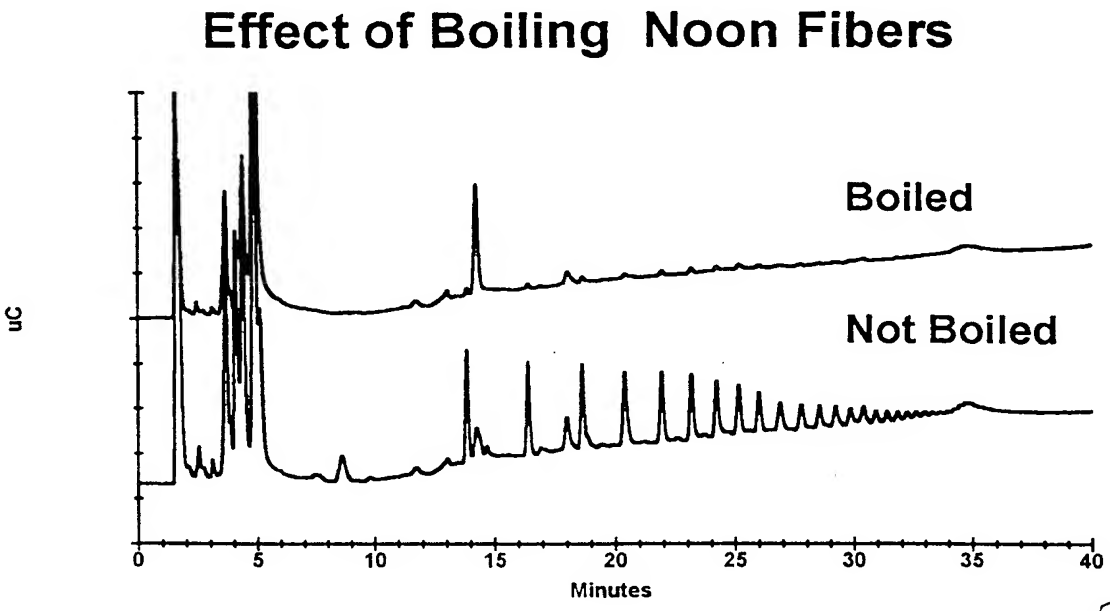


Fig 21a

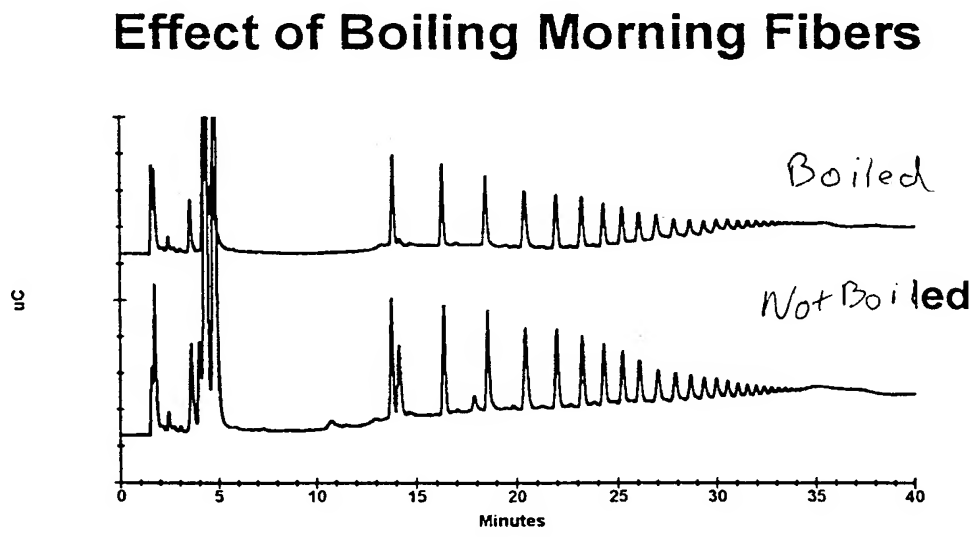


Fig 21b

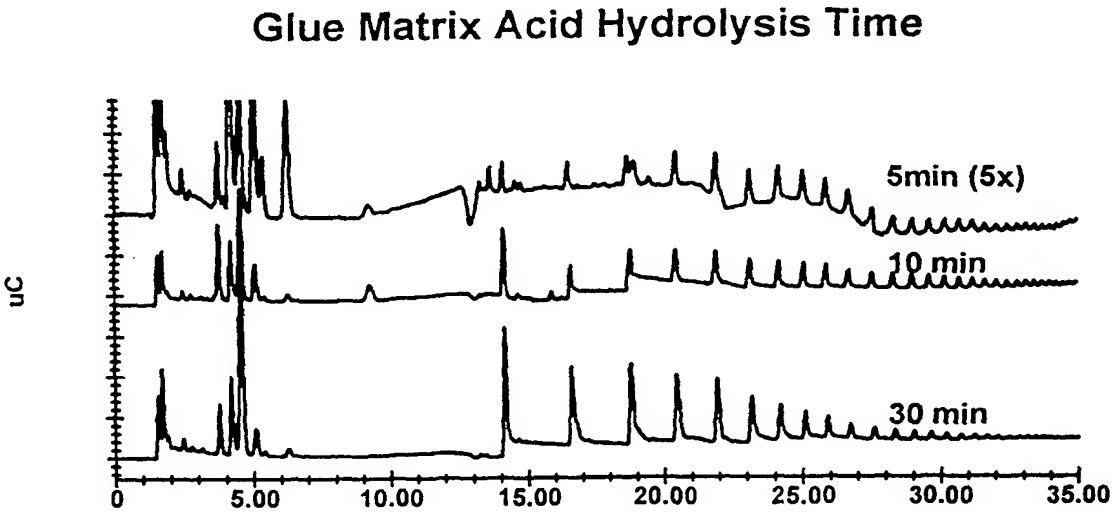

Glue Matrix Acid Hydrolysis Time
uC
5min (5x)
10 min
30 min
0
5.00
10.00
15.00
20.00
25.00
30.00
35.00
Minutes

Fig. 22

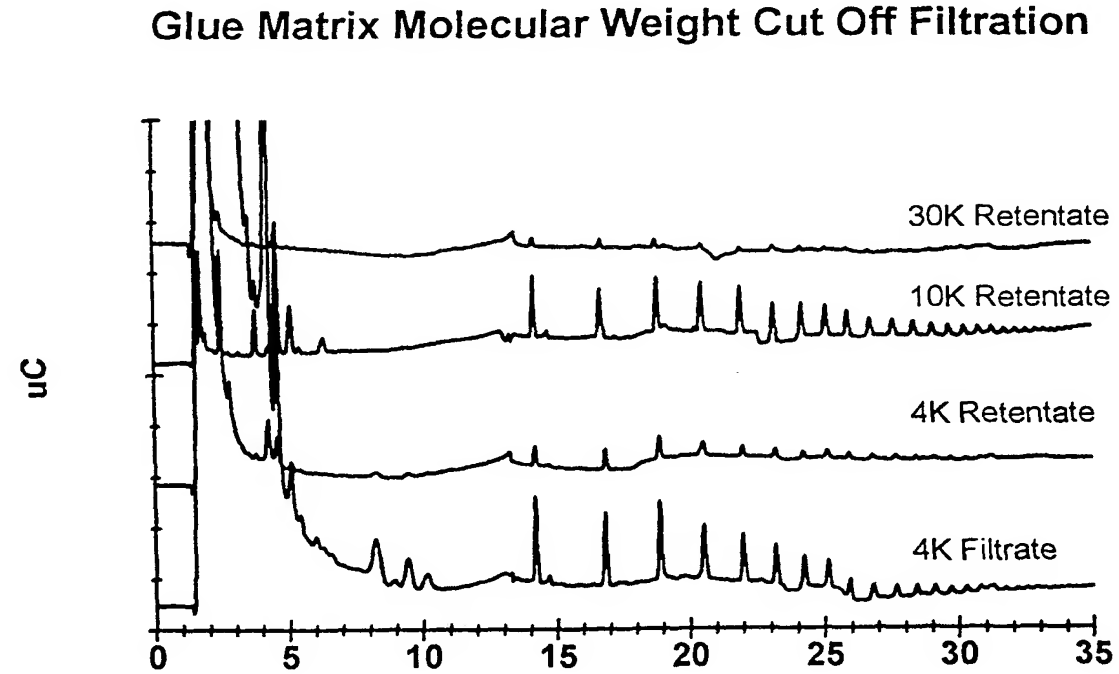

Glue Matrix Molecular Weight Cut Off Filtration
uC
30K Retentate
10K Retentate
4K Retentate
4K Filtrate
0
5
10
15
20
25
30
35
Minutes

Fig. 23

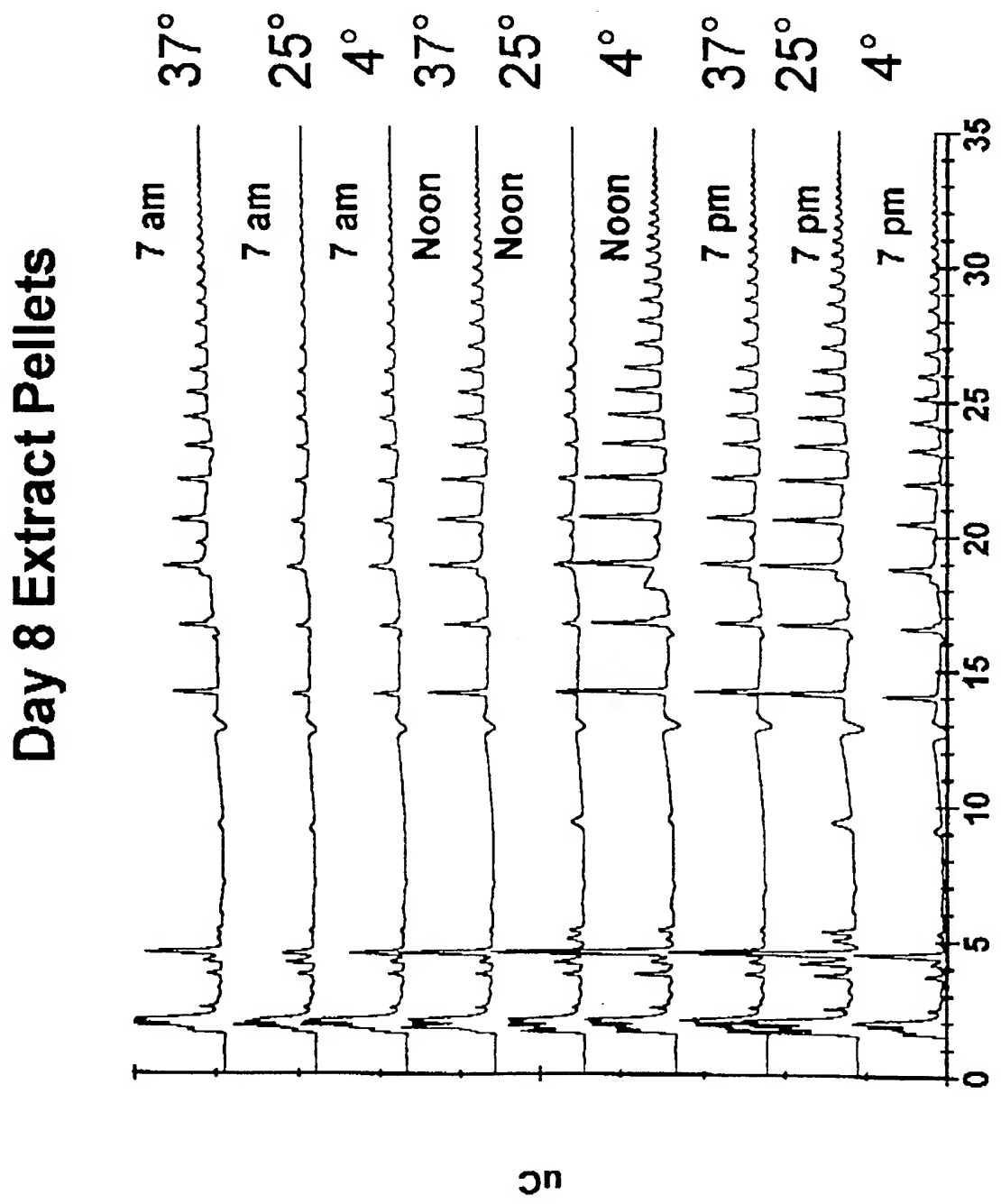
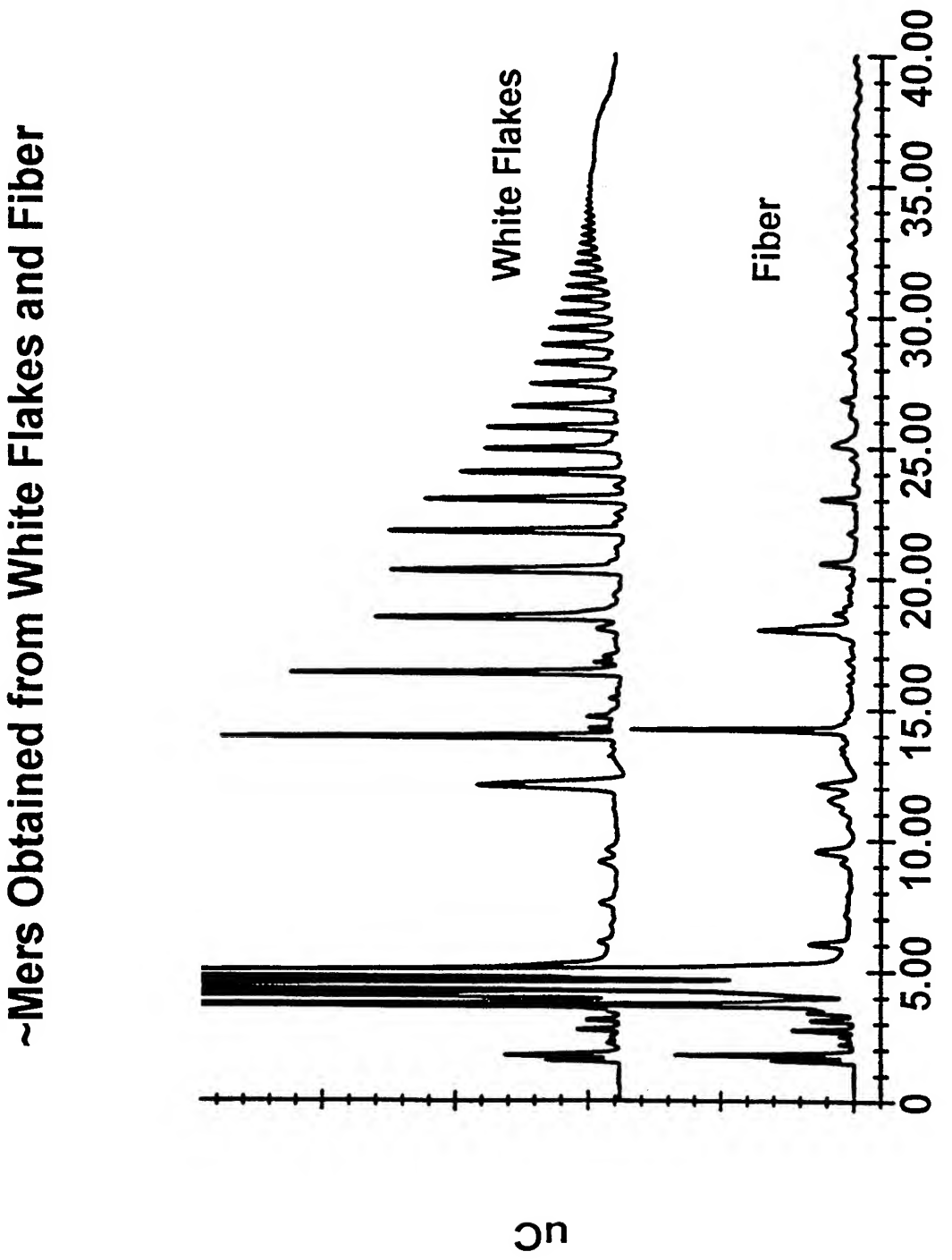

Day 8 Extract Pellets
7 am
37°
7 am
25°
7 am
4°
Noon
37°
Noon
25°
Noon
4°
7 pm
37°
7 pm
25°
7 pm
4°
0
5
10
15
20
25
30
35
Minutes
nC

Fig. 24


~Mers Obtained from White Flakes and Fiber
White Flakes
Fiber
0
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
Minutes
uC
Fig. 25

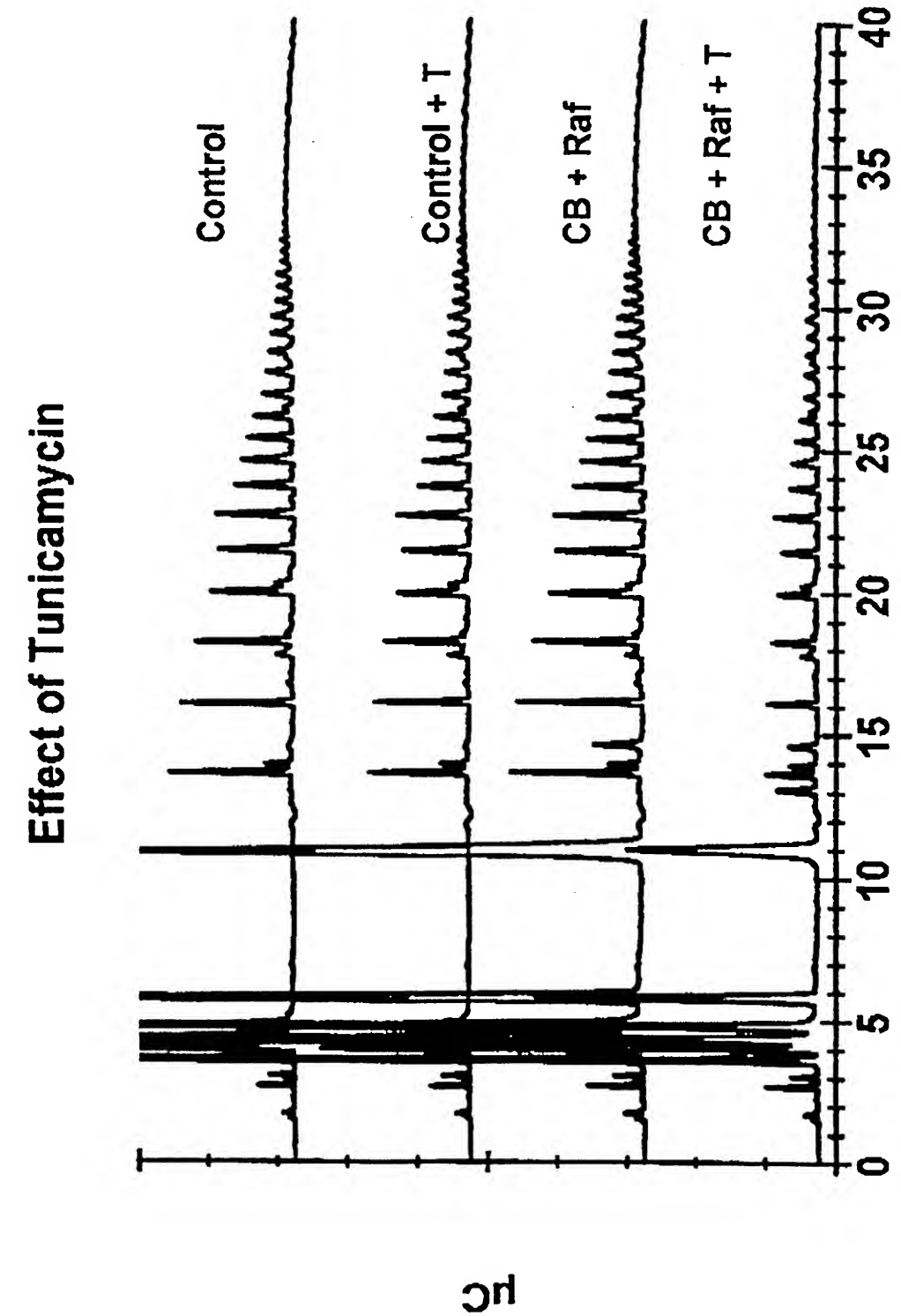

Effect of Tunicamycin
Control
Control + T
CB + Raf
CB + Raf + T
0
5
10
15
20
25
30
35
40
Minutes
nC

Fig. 26

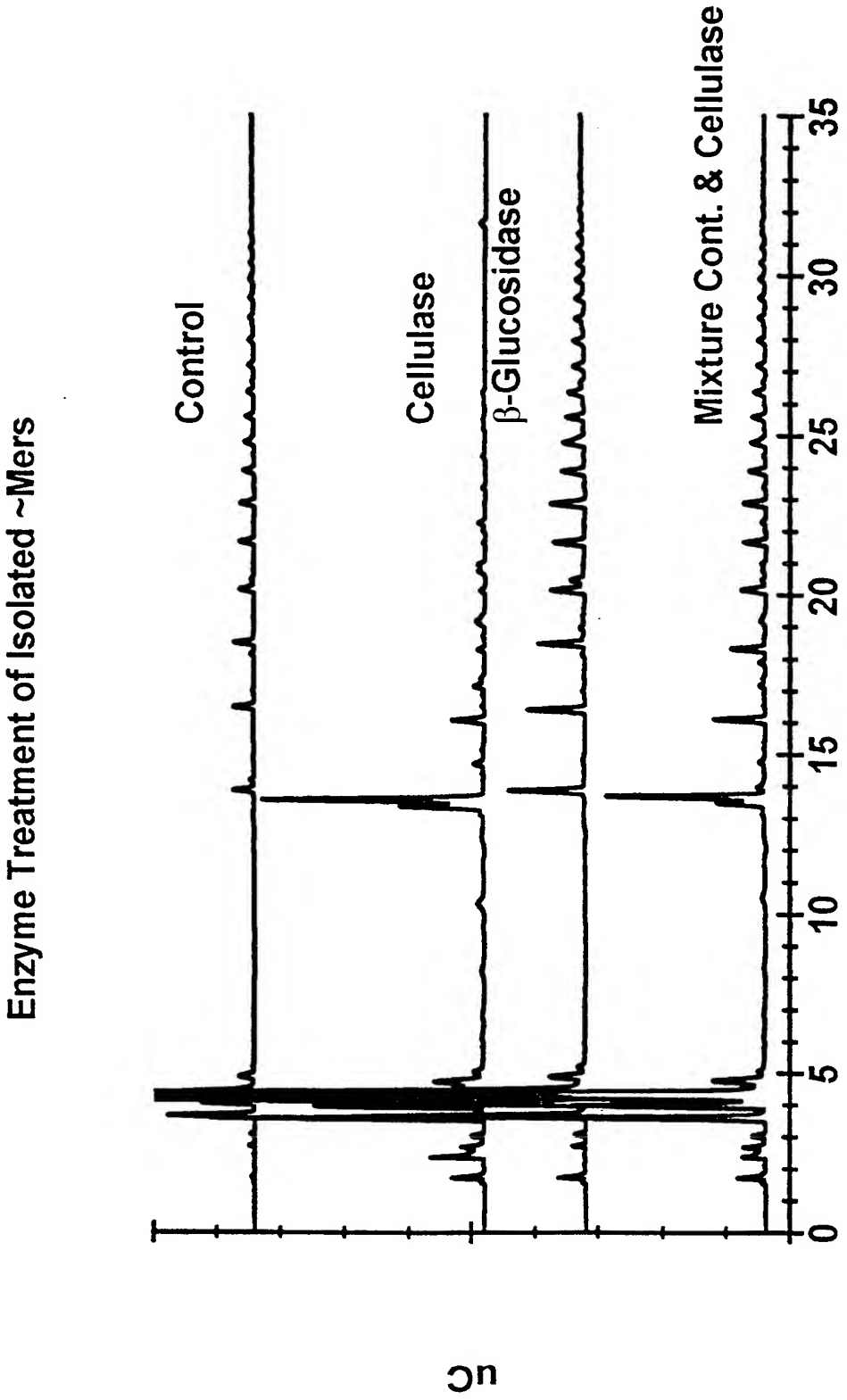

Enzyme Treatment of Isolated ~Mers
Control
Cellulase
β-Glucosidase
Mixture Cont. & Cellulase
nC
0
5
10
15
20
25
30
35
Minutes

Fig. 27

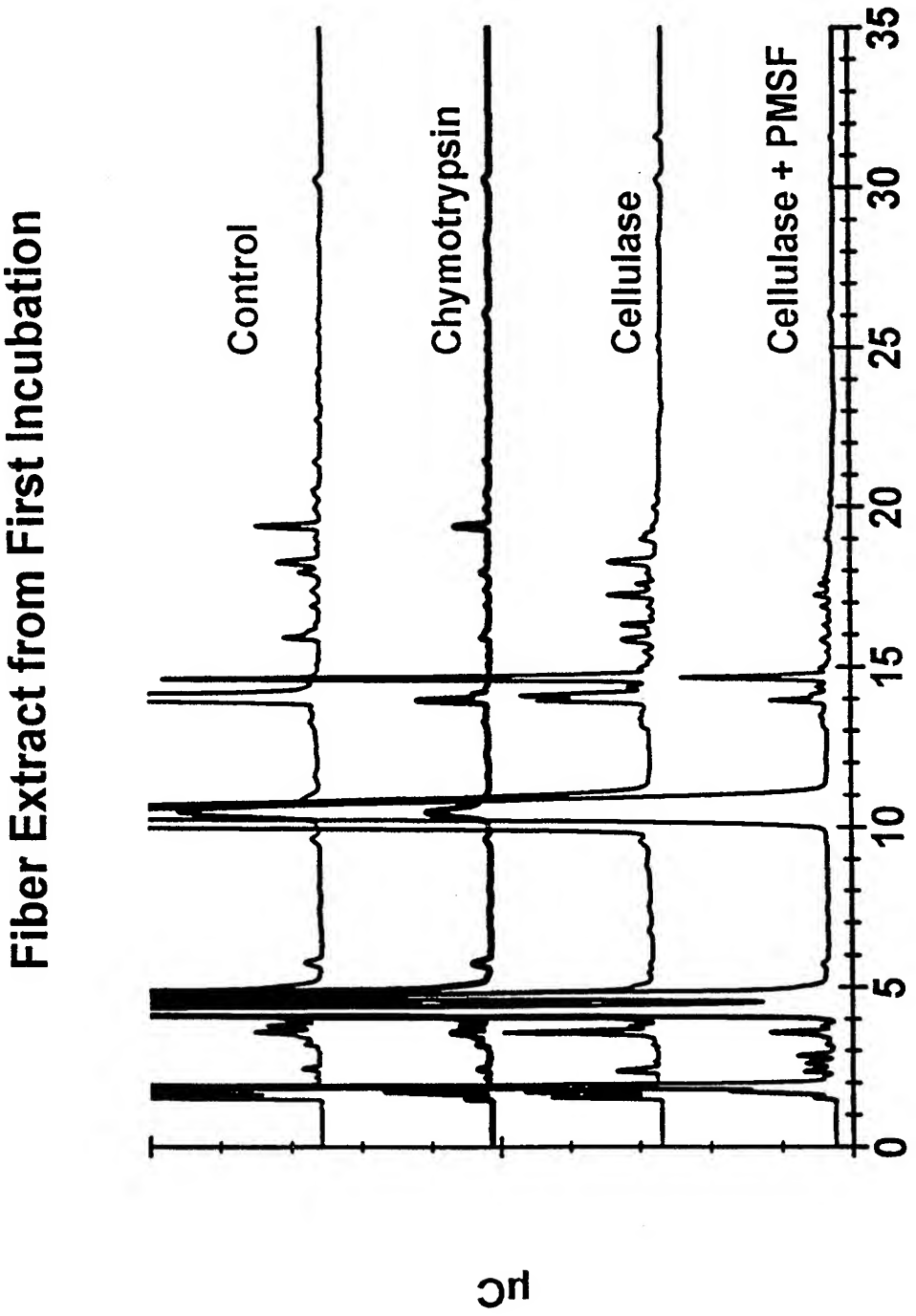

Fiber Extract from First Incubation
Control
Chymotrypsin
Cellulase
Cellulase + PMSF
0
5
10
15
20
25
30
35
Minutes
nC

Fig. 28

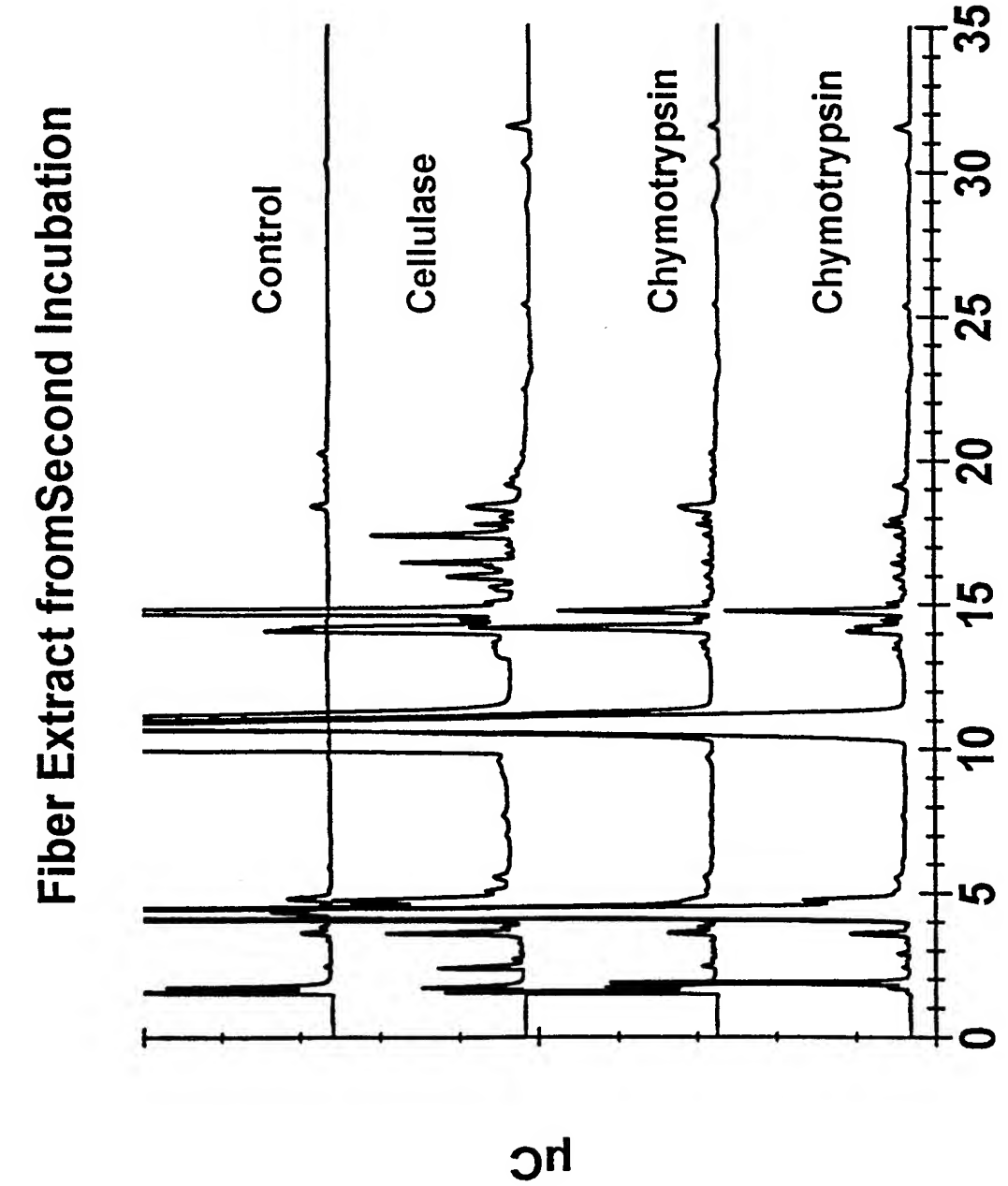

Fiber Extract fromSecond Incubation
Control
Cellulase
Chymotrypsin
Chymotrypsin
0
5
10
15
20
25
30
35
Minutes
HC

Fig. 29

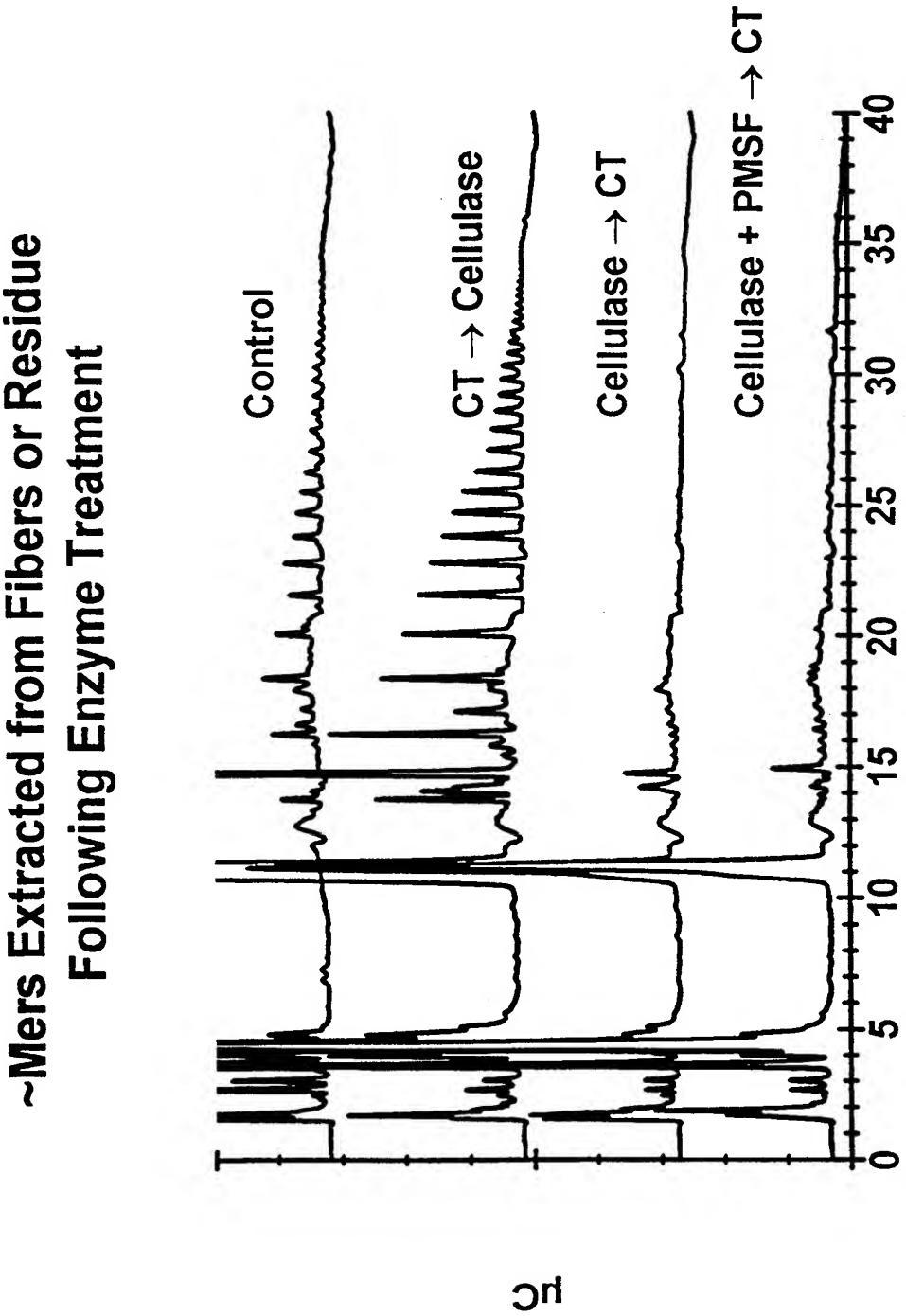

~Mers Extracted from Fibers or Residue
Following Enzyme Treatment
Control
CT → Cellulase
Cellulase → CT
Cellulase + PMSF → CT
0
5
10
15
20
25
30
35
40
Minutes
nC

Fig. 30

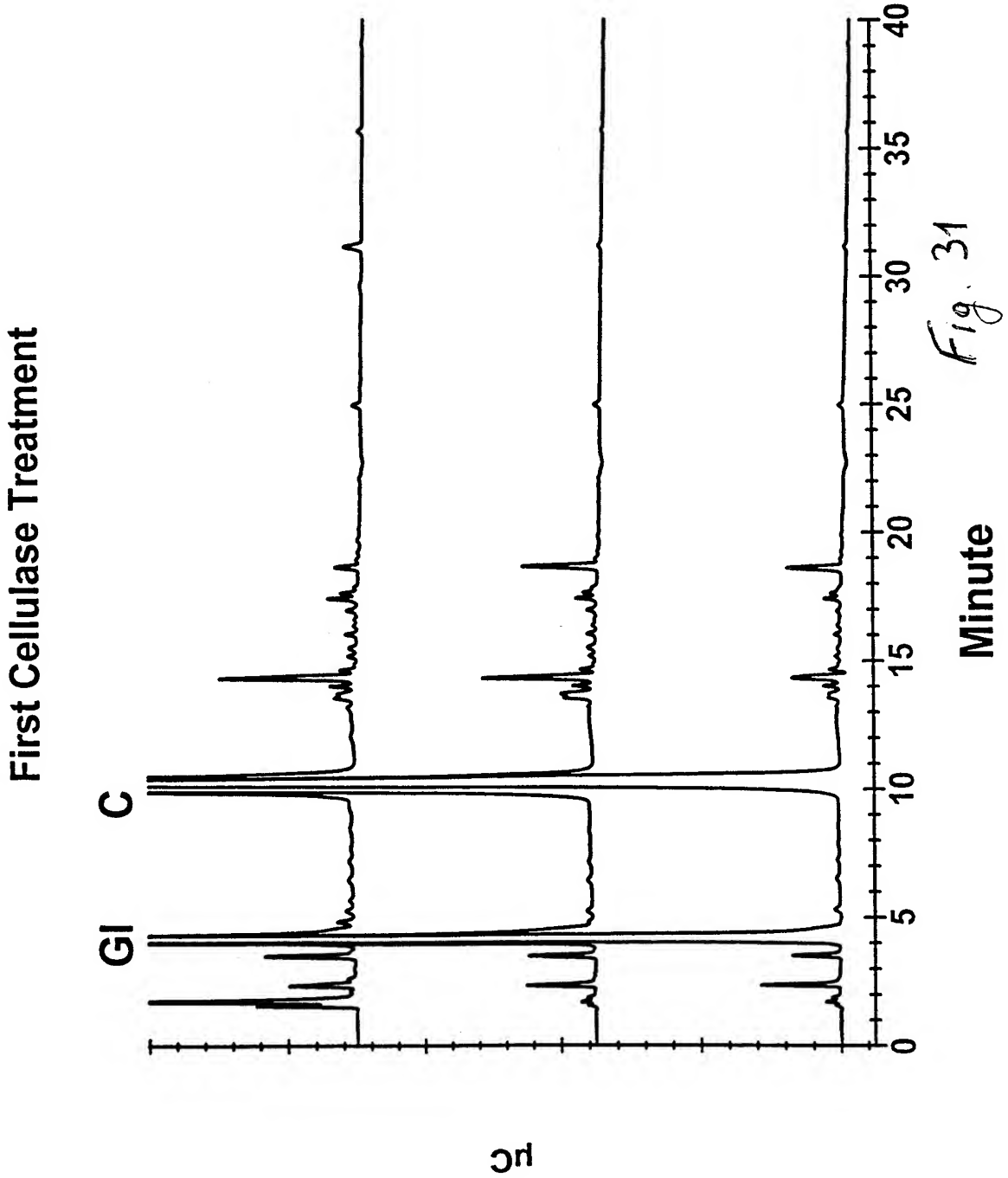

First Cellulase Treatment
GI
C
0
5
10
15
20
25
30
35
40
Minute
nC

Fig. 31

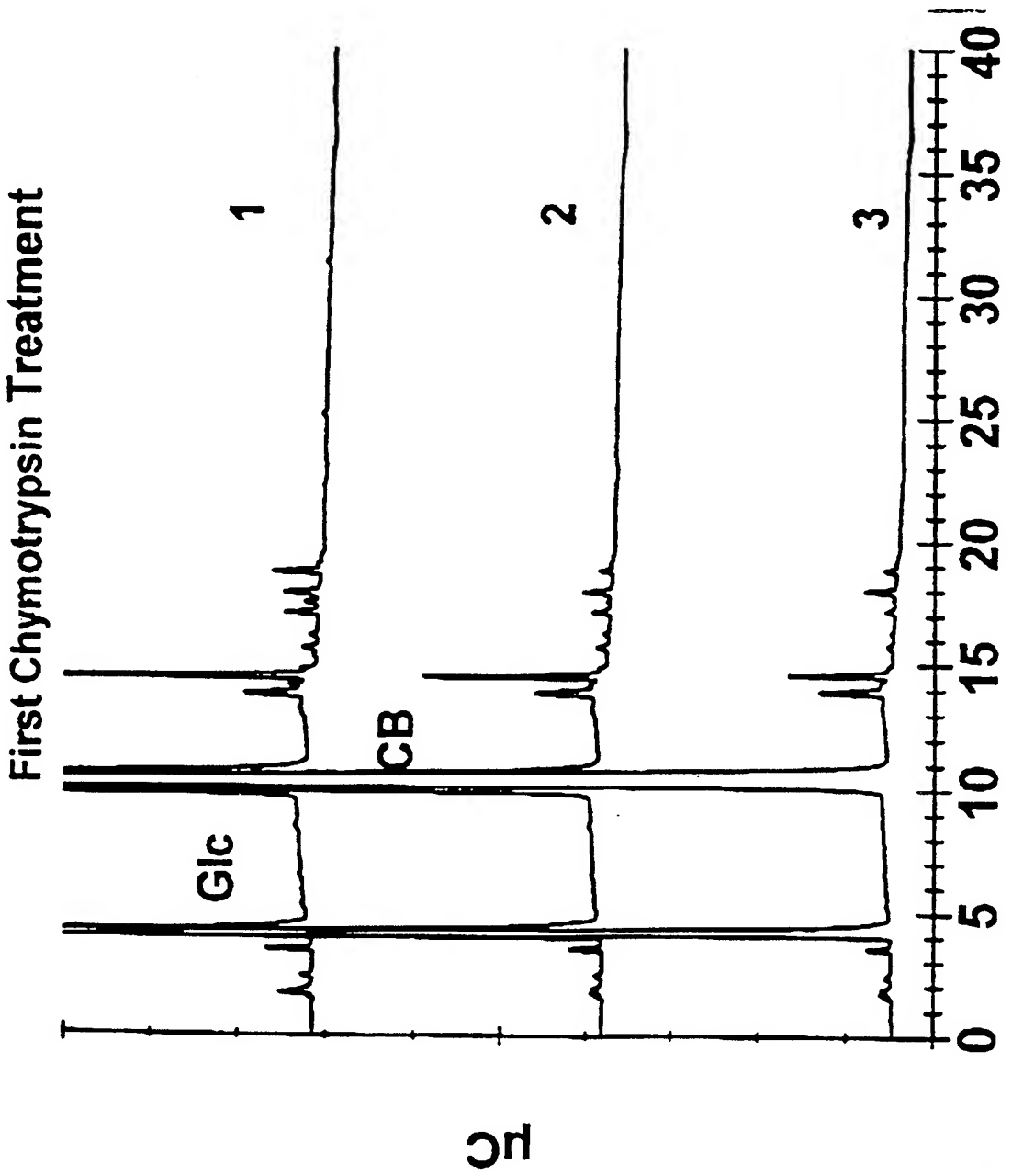

First Chymotrypsin Treatment
1
2
3
Glc
CB
0
5
10
15
20
25
30
35
40
Minutes
HC

Fig 32

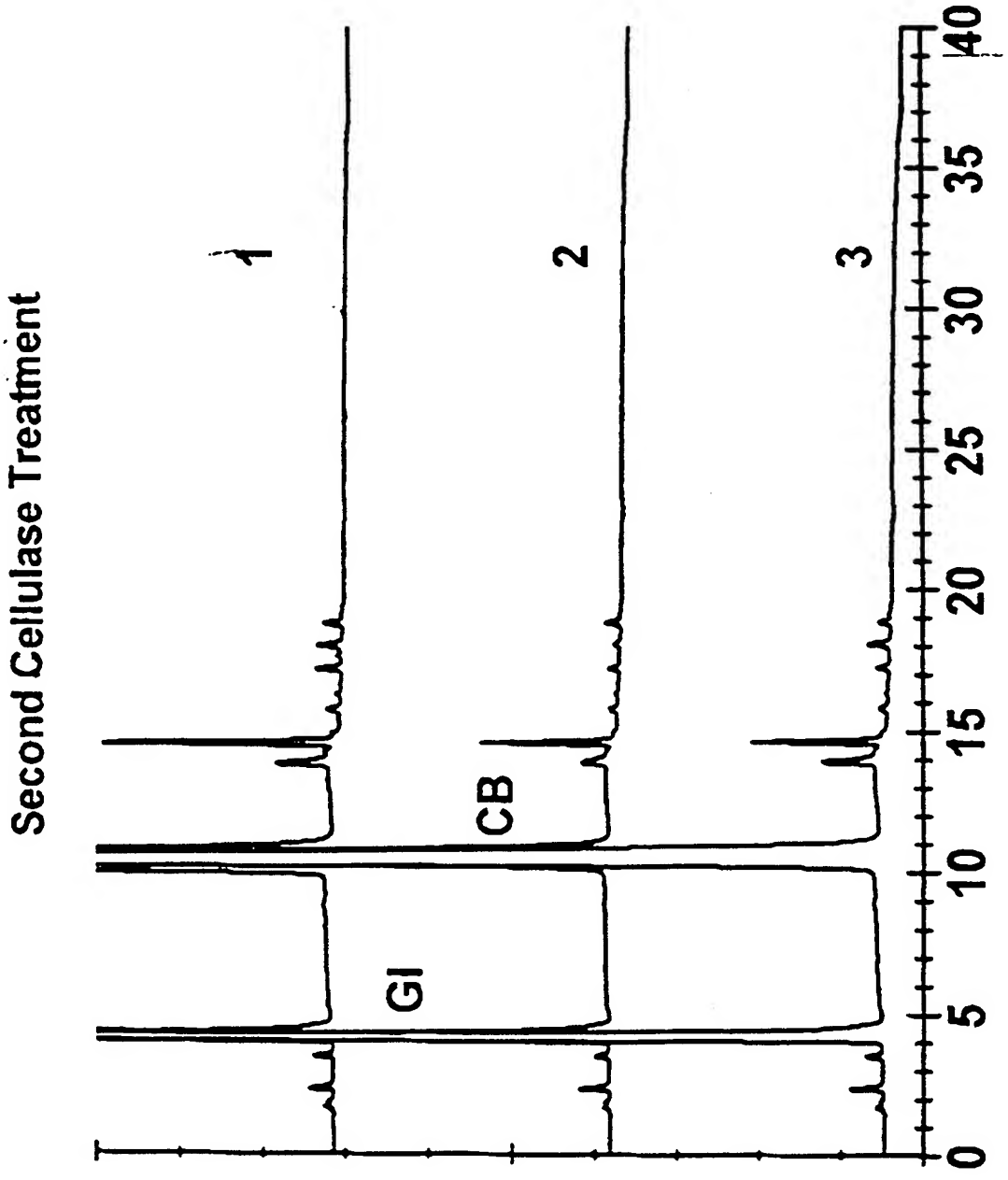

Second Cellulase Treatment
1
2
3
GI
CB
0
5
10
15
20
25
30
35
40
Minutes
RC

Fig. 33

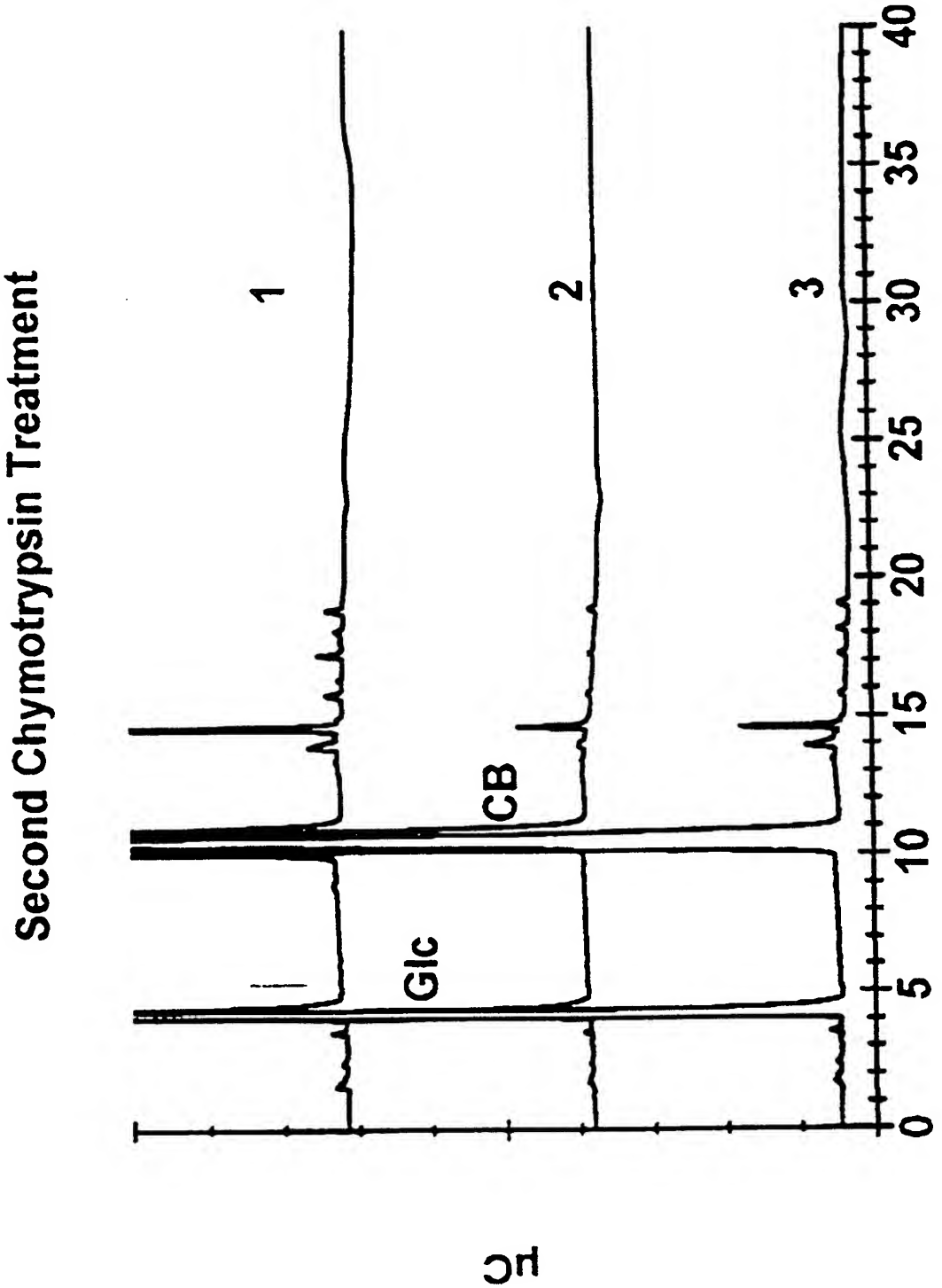

Second Chymotrypsin Treatment
1
2
3
Glc
CB
0
5
10
15
20
25
30
35
40
Minutes
HC

Fig 34

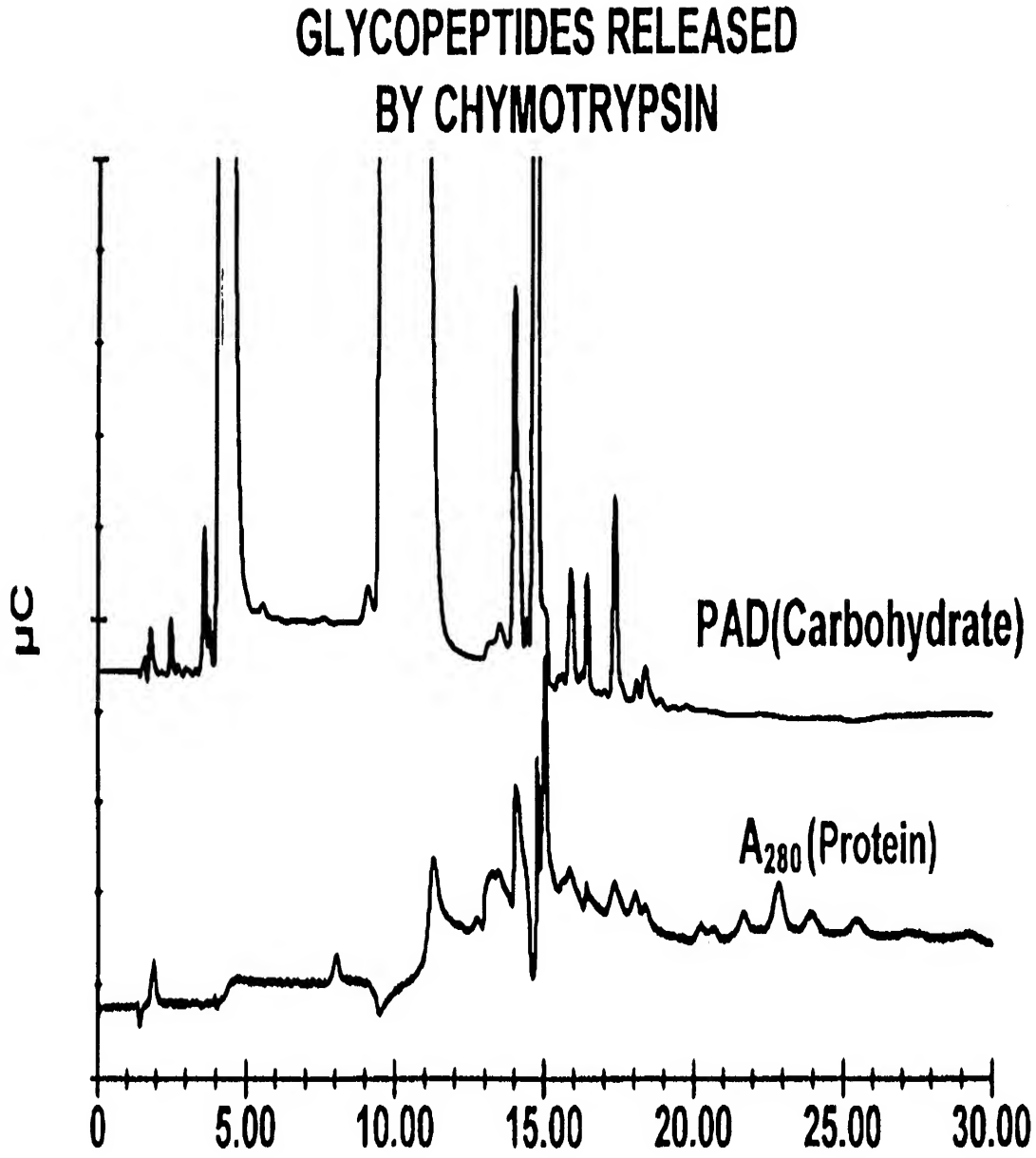

GLYCOPEPTIDES RELEASED
BY CHYMOTRYPSIN
nC
PAD(Carbohydrate)
A280(Protein)
0
5.00
10.00
15.00
20.00
25.00
30.00
Minutes

Fig 35

MONOSACCHARIDE COMPOSITION WHITE PARTICLES

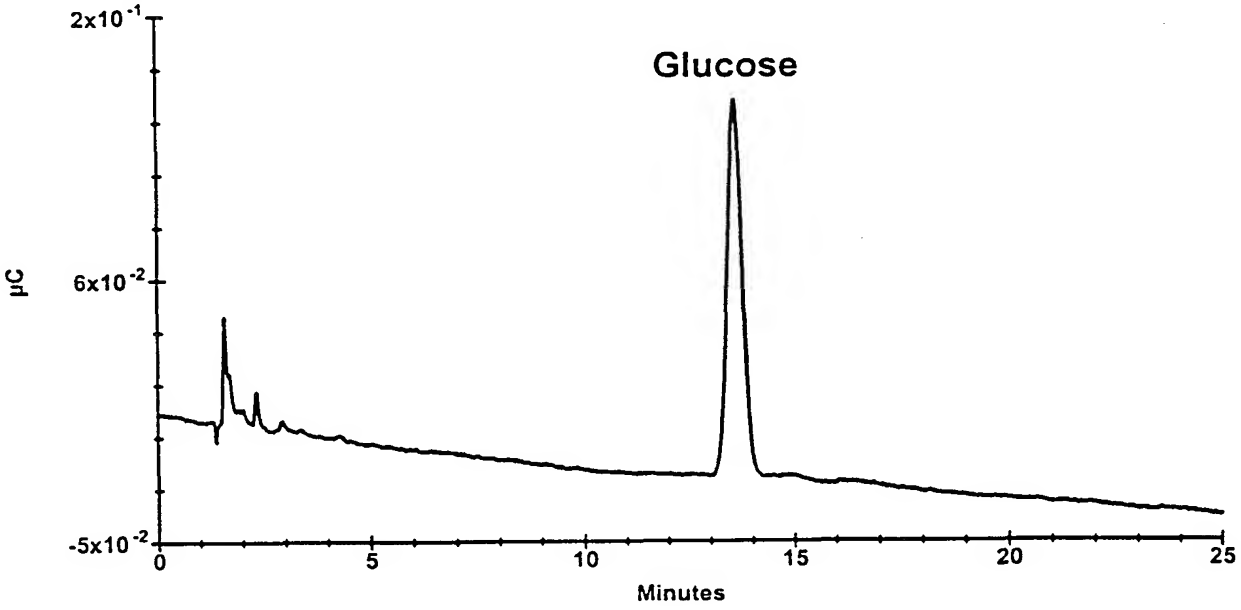


Fig. 36